US009297041B1

(12) **United States Patent**
Benner

(10) **Patent No.: US 9,297,041 B1**
(45) **Date of Patent: Mar. 29, 2016**

(54) **INEXPENSIVE AUTONOMOUS ASSEMBLY OF ULTRA-LARGE (UL) DNA CONSTRUCTS**

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(72) Inventor: **Steven Albert Benner**, Gainesville, FL (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 65 days.

(21) Appl. No.: **14/048,671**

(22) Filed: **Oct. 8, 2013**

Related U.S. Application Data

(63) Continuation-in-part of application No. 13/936,309, filed on Jul. 8, 2013, now abandoned.

(60) Provisional application No. 61/669,295, filed on Jul. 9, 2012.

(51) **Int. Cl.**
C12Q 1/68 (2006.01)
C12P 19/34 (2006.01)

(52) **U.S. Cl.**
CPC ***C12Q 1/6862*** (2013.01); ***C12P 19/34*** (2013.01); *C12Q 2521/501* (2013.01); *C12Q 2525/117* (2013.01)

(58) **Field of Classification Search**
CPC C12Q 1/6862; C12Q 2521/501; C12Q 2525/117
USPC 435/6.1, 91.1, 91.2
See application file for complete search history.

(56) **References Cited**

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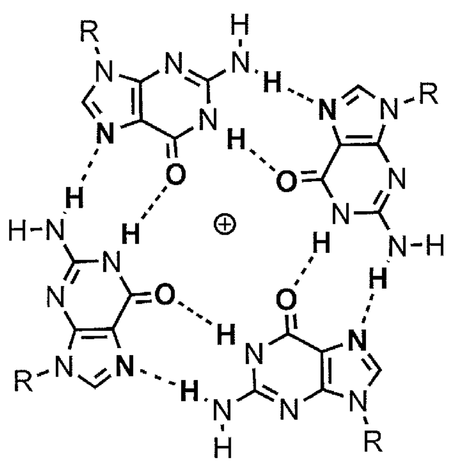
U.S. Appl. No. 12/653,613, filed Dec. 16, 2009, Steven A. Benner.

Primary Examiner — Jezia Riley

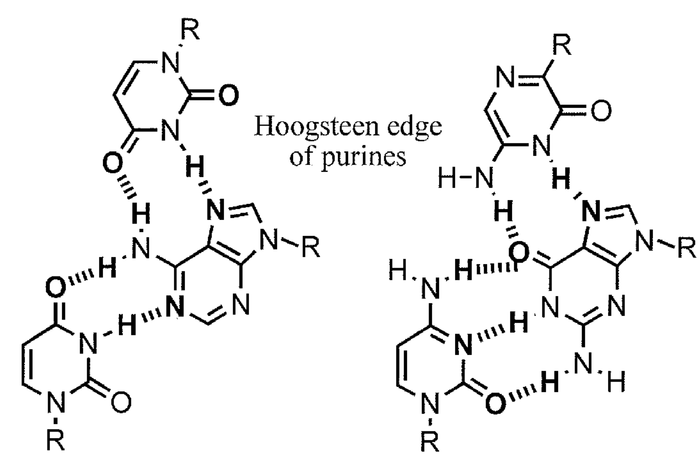
(57) **ABSTRACT**

This invention provides processes to assemble many (greater than 20) partially overlapping single stranded DNA molecules (fragments) having preselected sequences, followed by extension of those strands that hybridize at terminal overlap regions, and ligation of the extend products, creating a double-stranded DNA assembly. These processes use non-standard nucleotides carrying heterocyclic nucleobase analogs that implement non-standard hydrogen bonding patterns; these allow controlled annealing of the single stranded fragments via Watson-Crick rules, with less or no interference from a range of non-Watson Crick interactions, hairpin formations, or off-target hybridization displayed by standard nucleobases. This process includes an optional conversion step that replaces non-standard nucleobase pairs with standard nucleobase pairs, generating large synthetic DNA (LS-DNA) molecules containing only natural nucleotides. As useful application, this invention allows the assembly of genes encoding whole proteins (typically 1000-3000 nucleotide pairs) from a collection of single stranded DNA fragments at reduced cost and effort.

9 Claims, 12 Drawing Sheets



G-quartet
non-canonical, very stable fold



major groove "Hoogsteen" binding

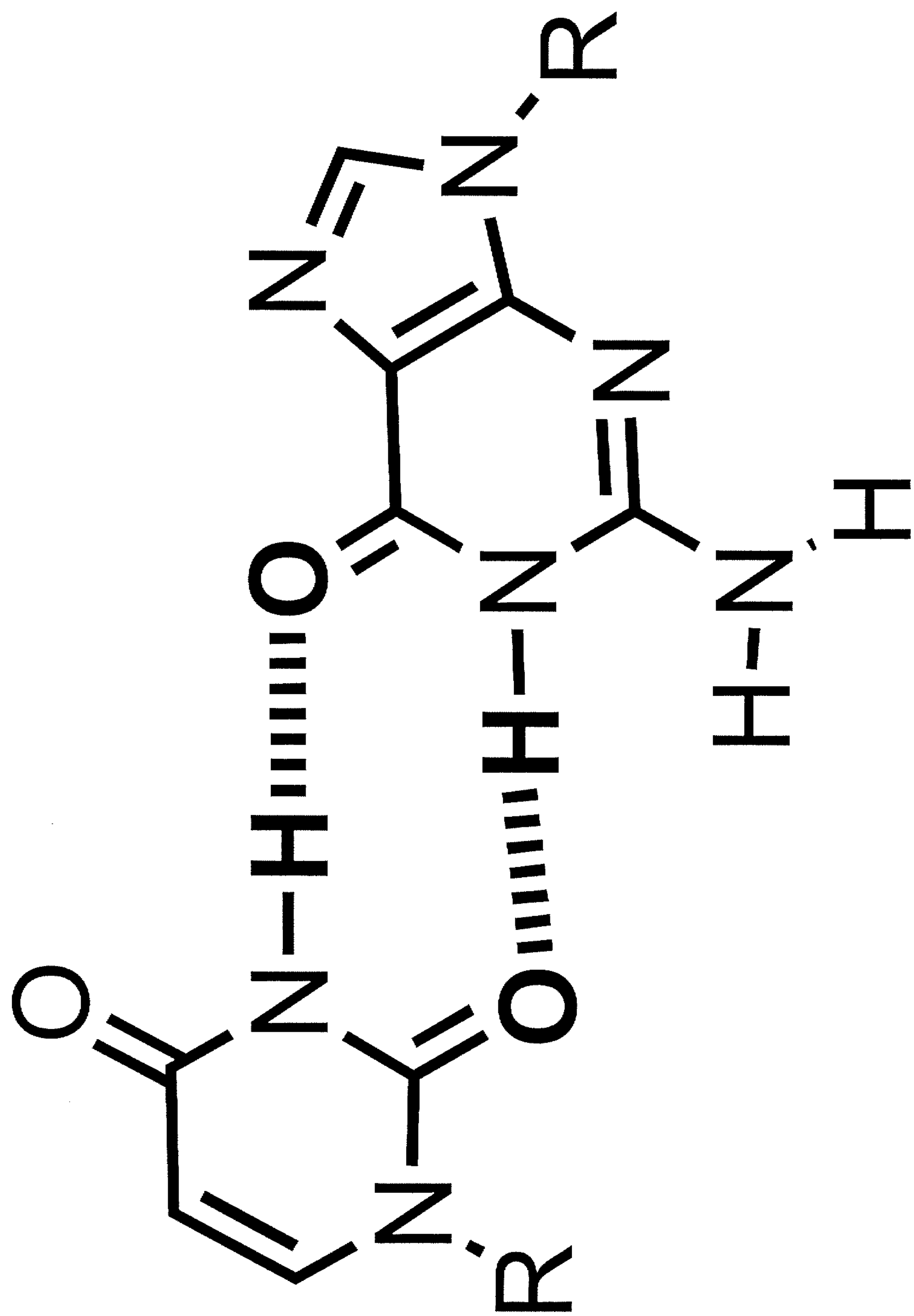


Figure 1

Figure 2

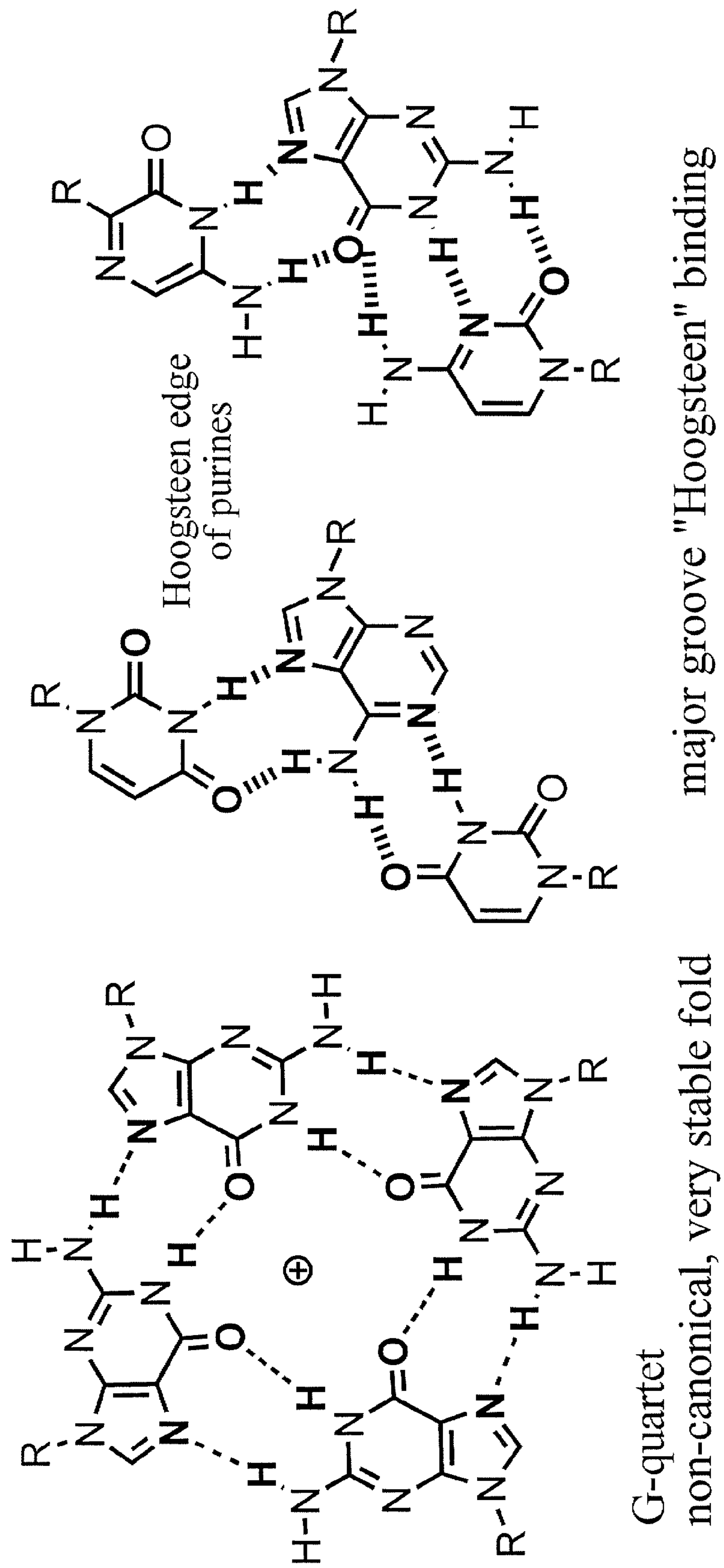

G-quartet
non-canonical, very stable fold
major groove "Hoogsteen" binding
Hoogsteen edge
of purines

Figure 3

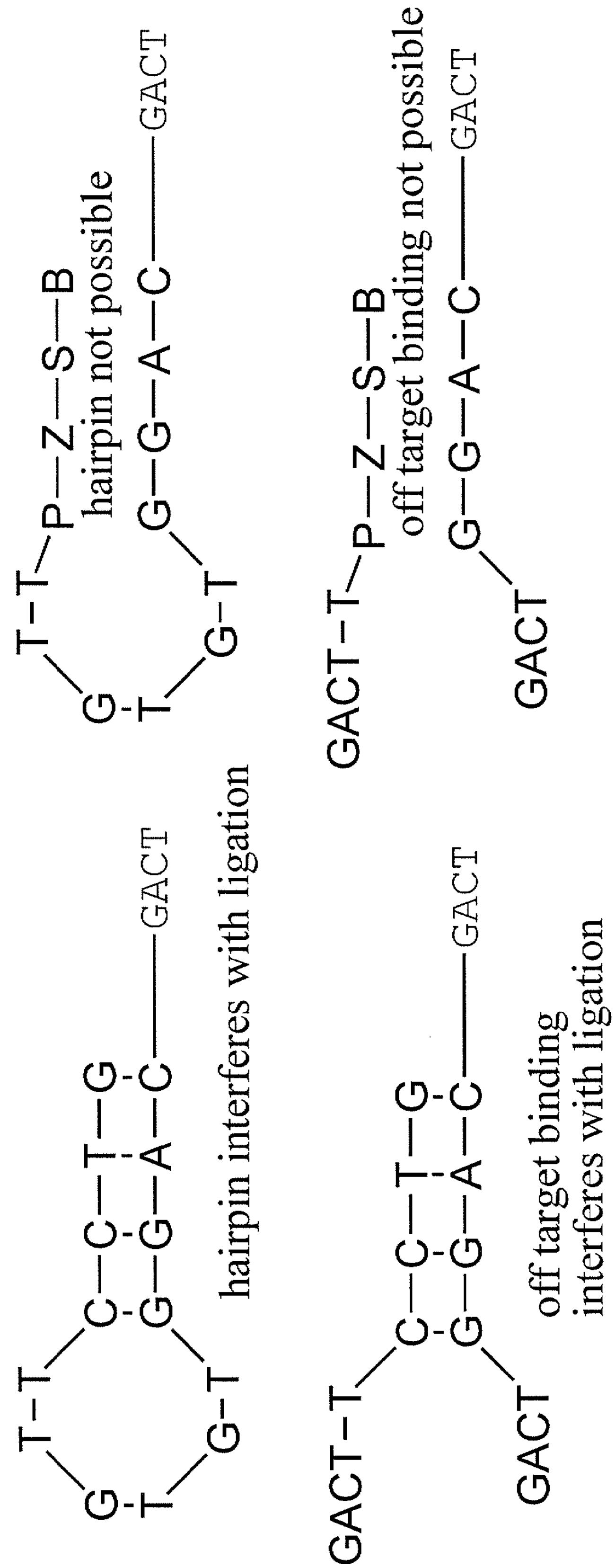

hairpin interferes with ligation
hairpin not possible
off target binding interferes with ligation
off target binding not possible
GACT
P—Z—S—B

Figure 4

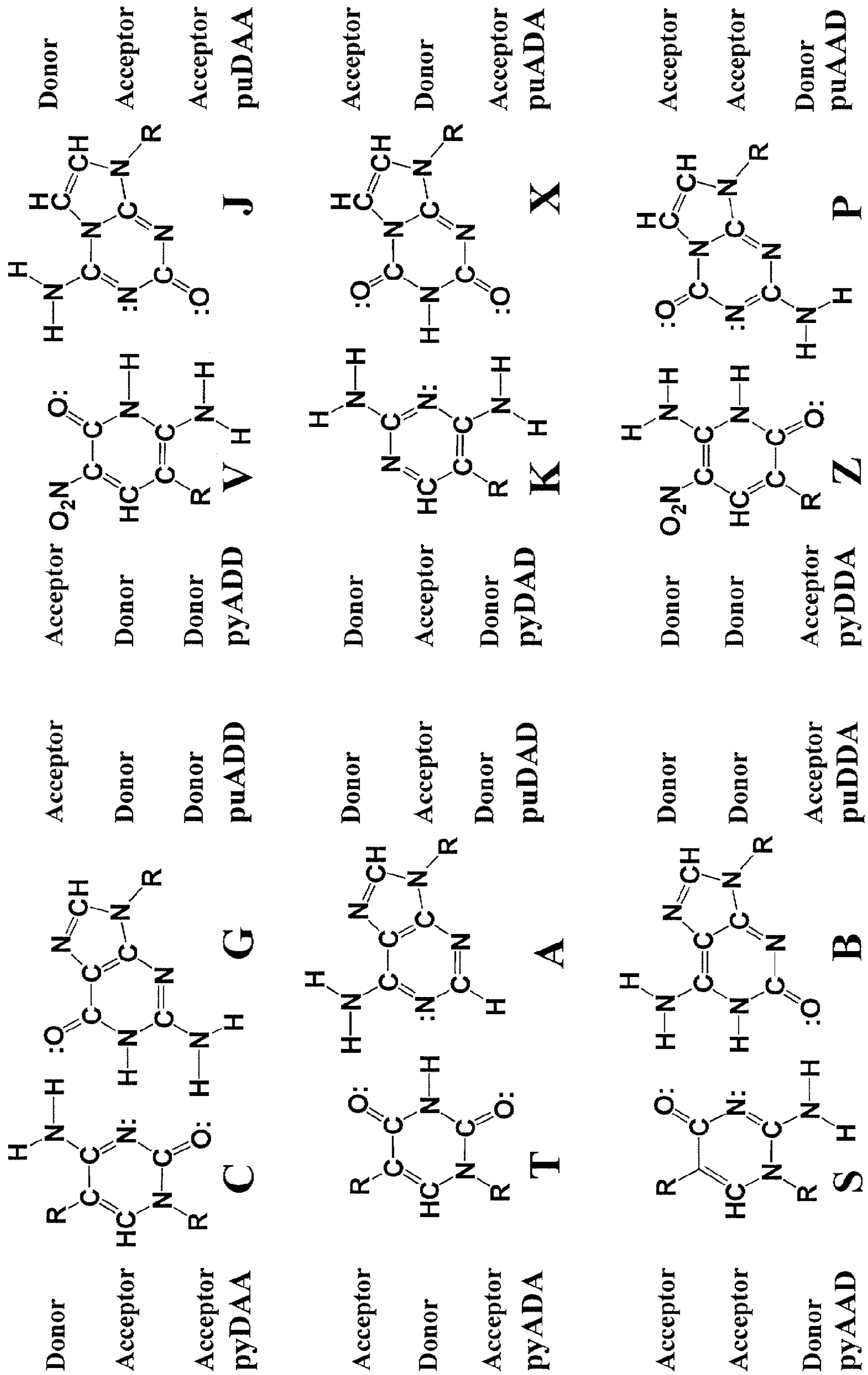

C
pyDAA
G
puADD
T
pyADA
A
puDAD
S
pyAAD
B
puDDA
V
pyADD
J
puDAA
K
pyDAD
X
puADA
Z
pyDDA
P
puAAD

Figure 5a

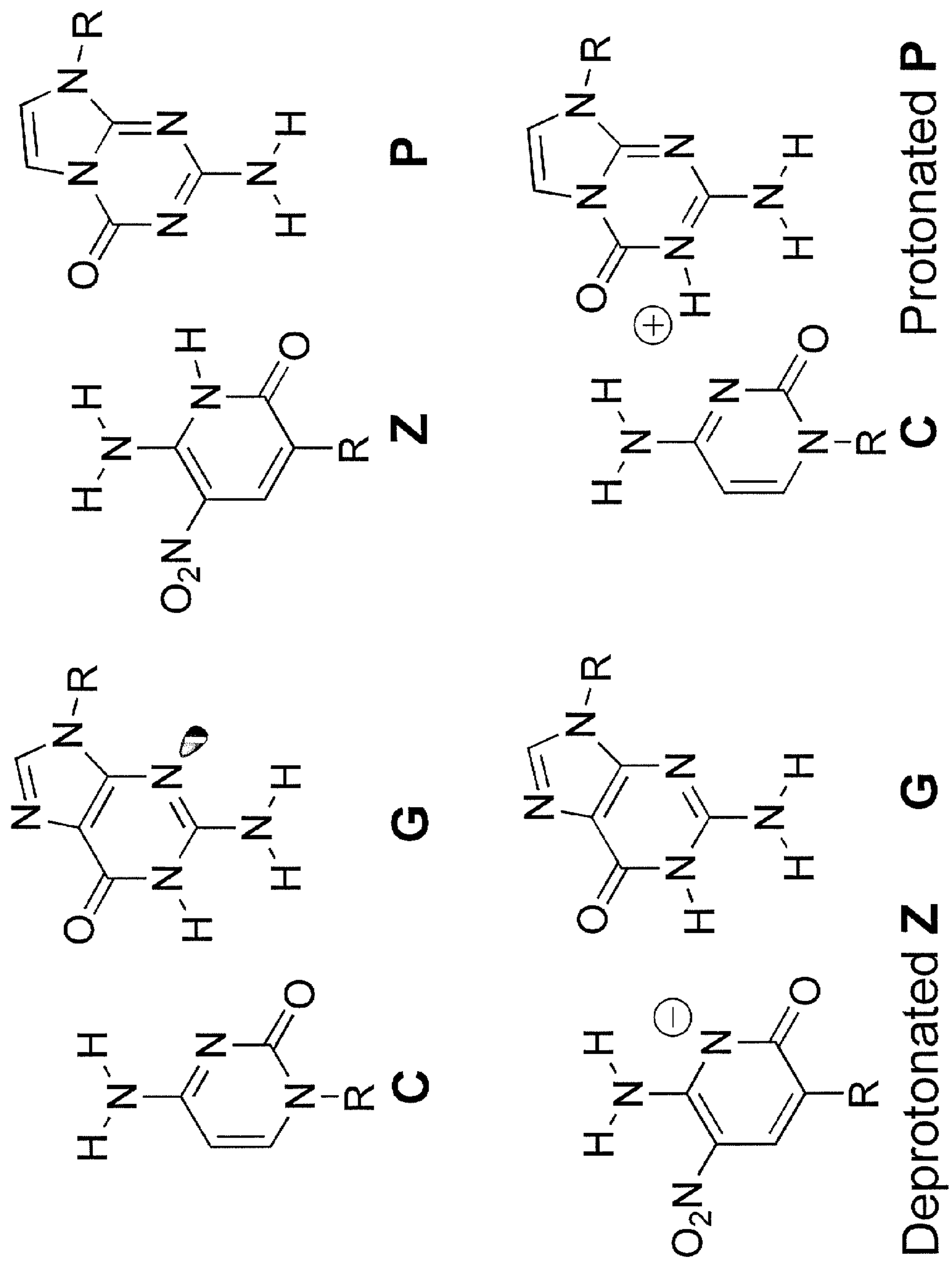


Figure 5b

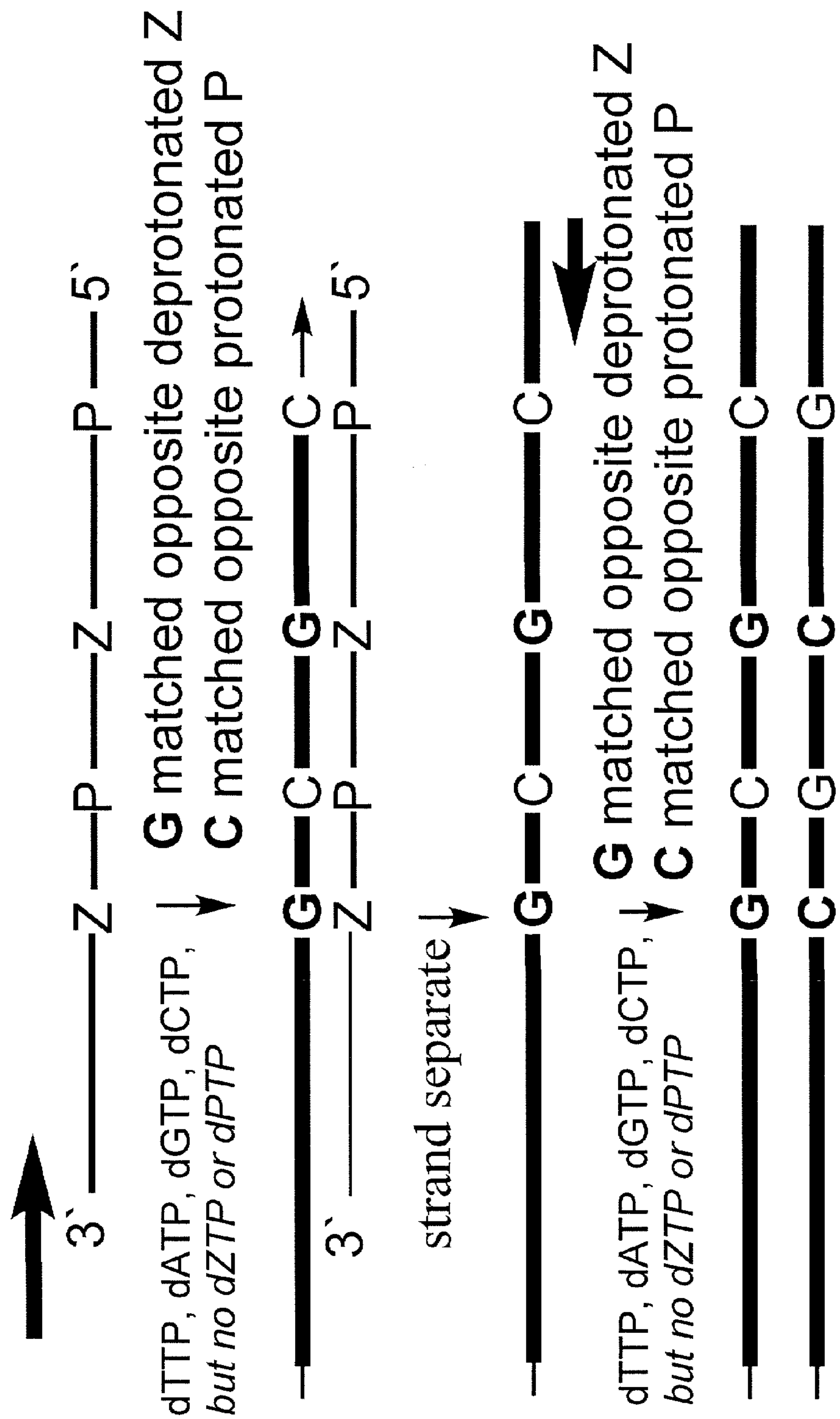

3′ Z P Z P 5′
dTTP, dATP, dGTP, dCTP,
but no dZTP or dPTP
G matched opposite deprotonated Z
C matched opposite protonated P
G C G C
3′ Z P Z P 5′
strand separate
G C G C
dTTP, dATP, dGTP, dCTP,
but no dZTP or dPTP
G matched opposite deprotonated Z
C matched opposite protonated P
G C G C
C G C G

Figure 6a

Donor

Donor B

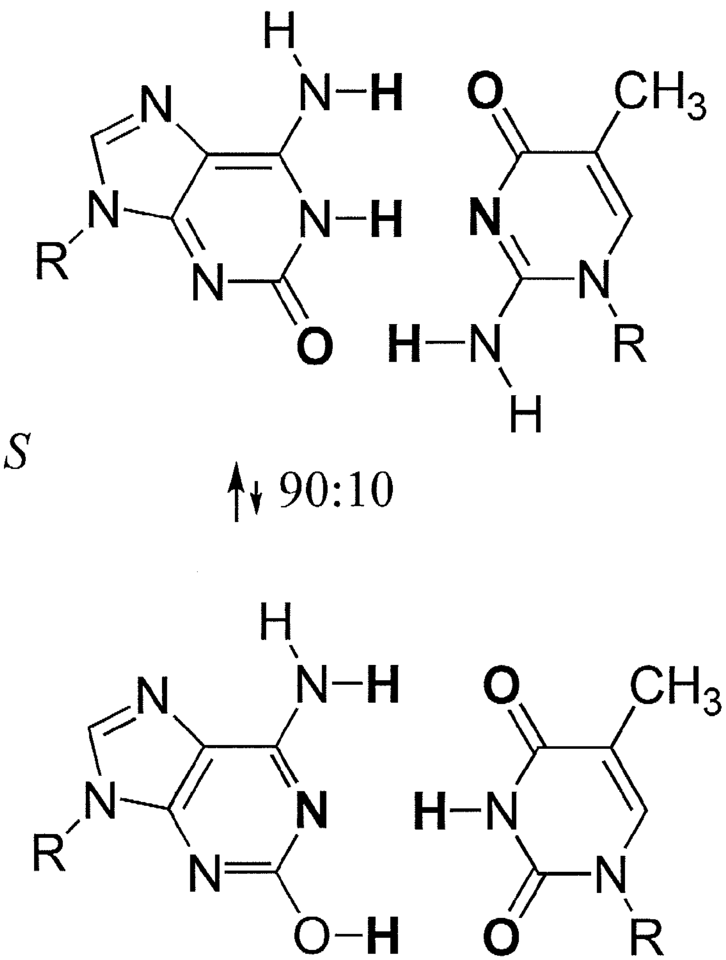
Acceptor

major keto form of B complements S

Acceptor

Acceptor S

Donor



Donor

Acceptor

Donor

minor enol form of B complements T

Acceptor

Donor T

Acceptor

Figure 6b

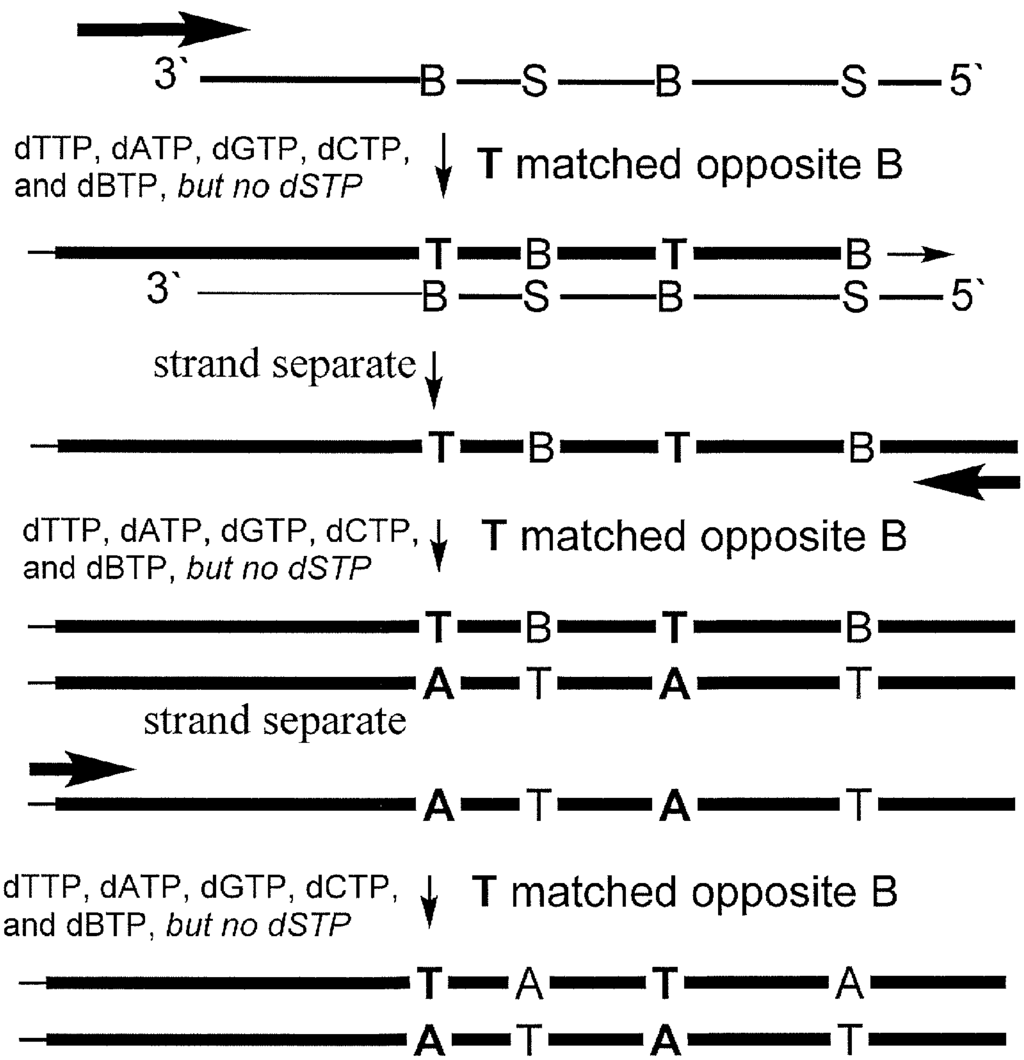

3`
B
S
B
S
5`
dTTP, dATP, dGTP, dCTP, and dBTP, *but no dSTP*
T matched opposite B
T
B
T
B
3`
B
S
B
S
5`
strand separate
T
B
T
B
dTTP, dATP, dGTP, dCTP, and dBTP, *but no dSTP*
T matched opposite B
T
B
T
B
A
T
A
T
strand separate
A
T
A
T
dTTP, dATP, dGTP, dCTP, and dBTP, *but no dSTP*
T matched opposite B
T
A
T
A
A
T
A
T

Figure 7

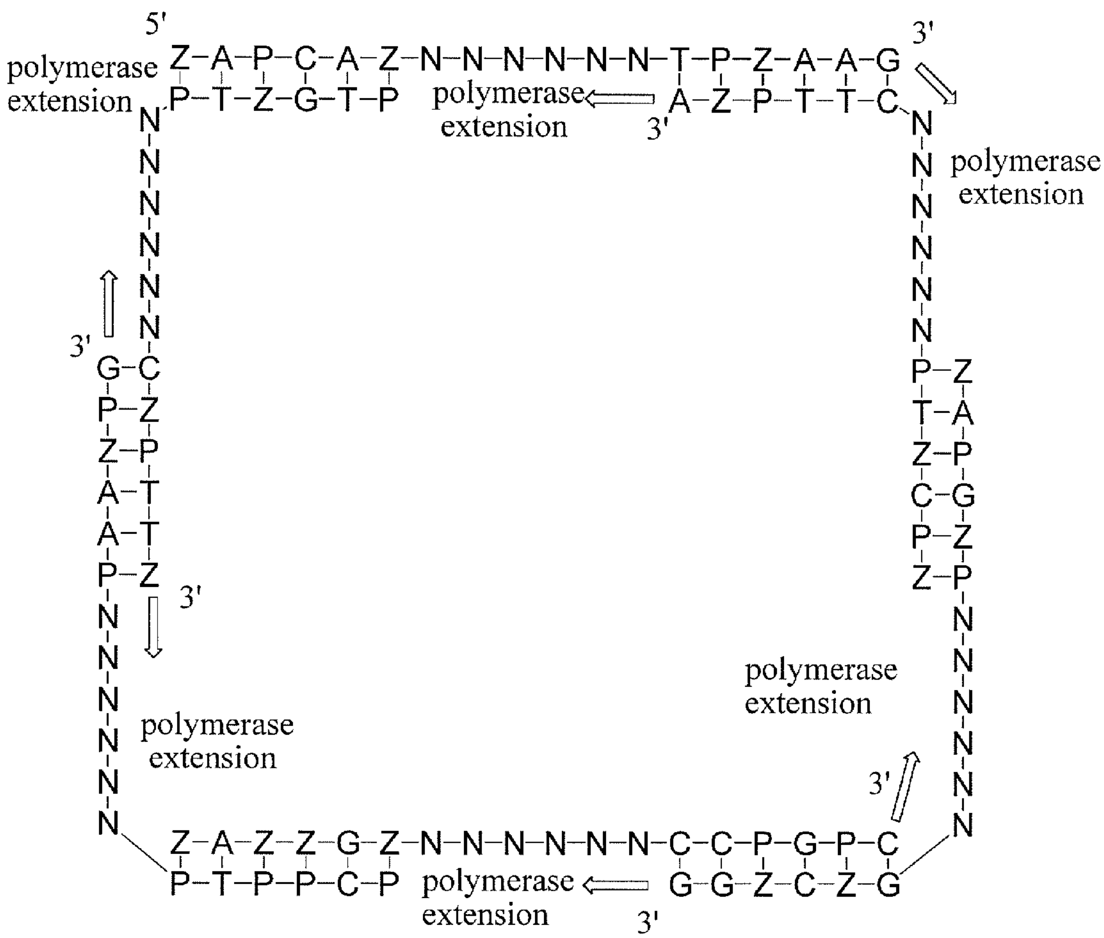

5'
Z-A-P-C-A-Z-N-N-N-N-N-N-T-P-Z-A-A-G
3'
P-T-Z-G-T-P
A-Z-P-T-T-C
3'
polymerase extension
polymerase extension
polymerase extension
3'
G-C
P-Z
Z-P
A-T
A-T
P-Z
3'
polymerase extension
P-Z
T-A
Z-P
C-G
P-Z
Z-P
polymerase extension
3'
Z-A-Z-Z-G-Z-N-N-N-N-N-N-C-C-P-G-P-C
P-T-P-P-C-P
G-G-Z-C-Z-G
3'
polymerase extension

Figure 8

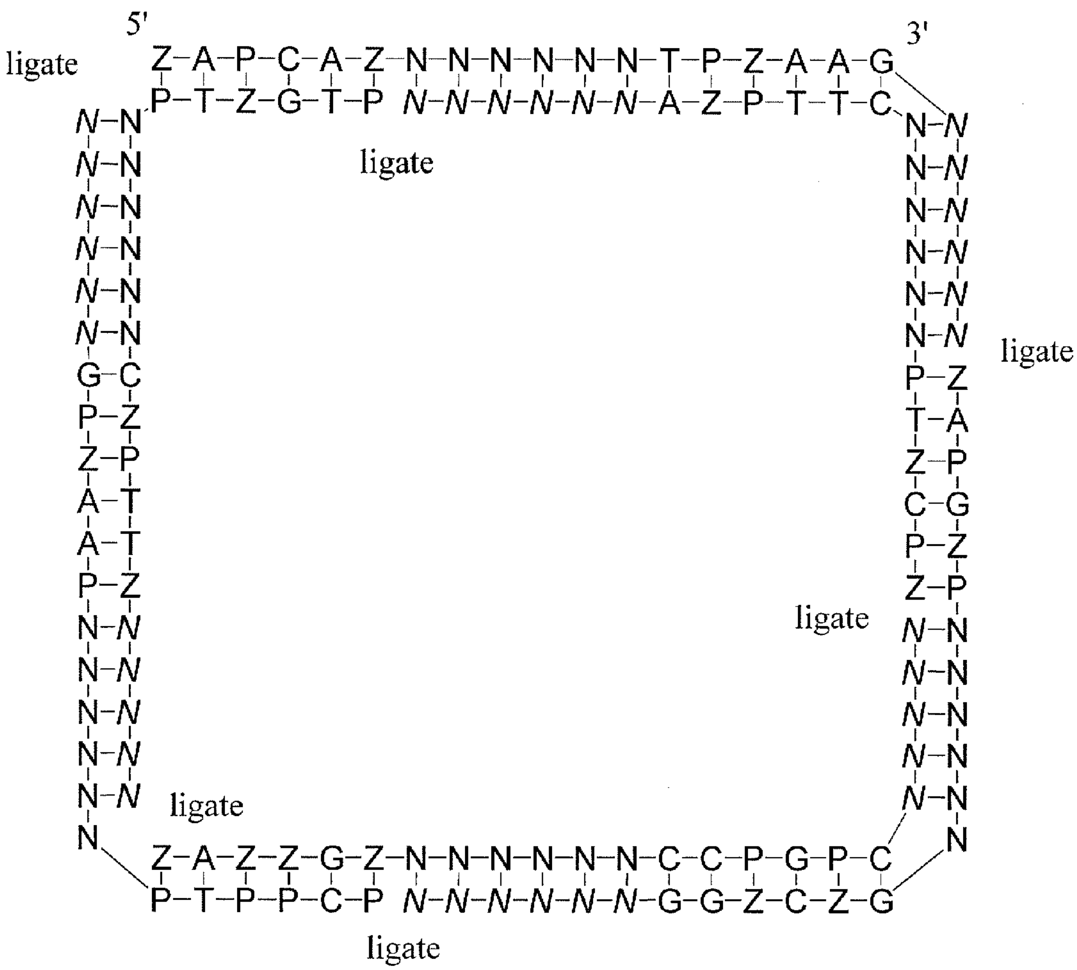


Figure 9

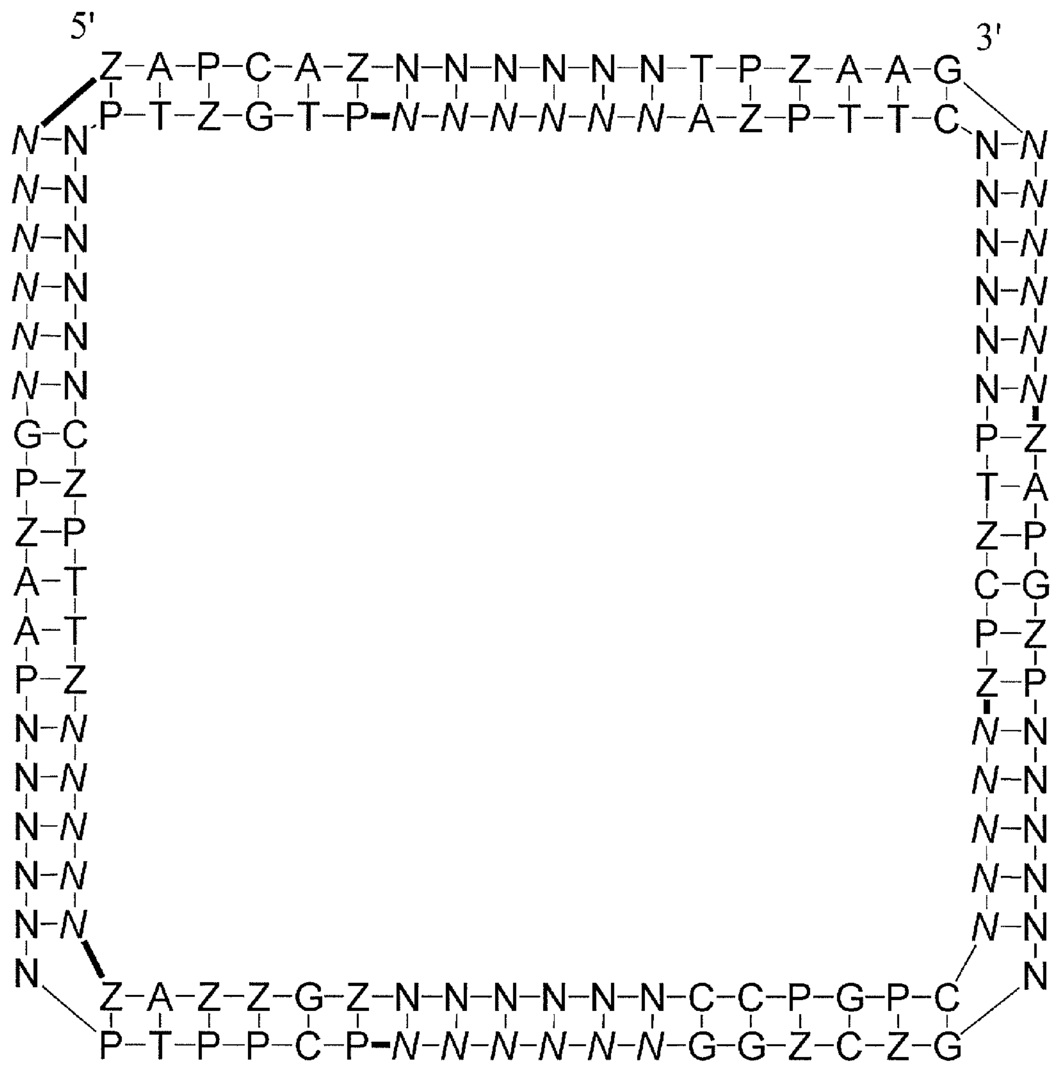
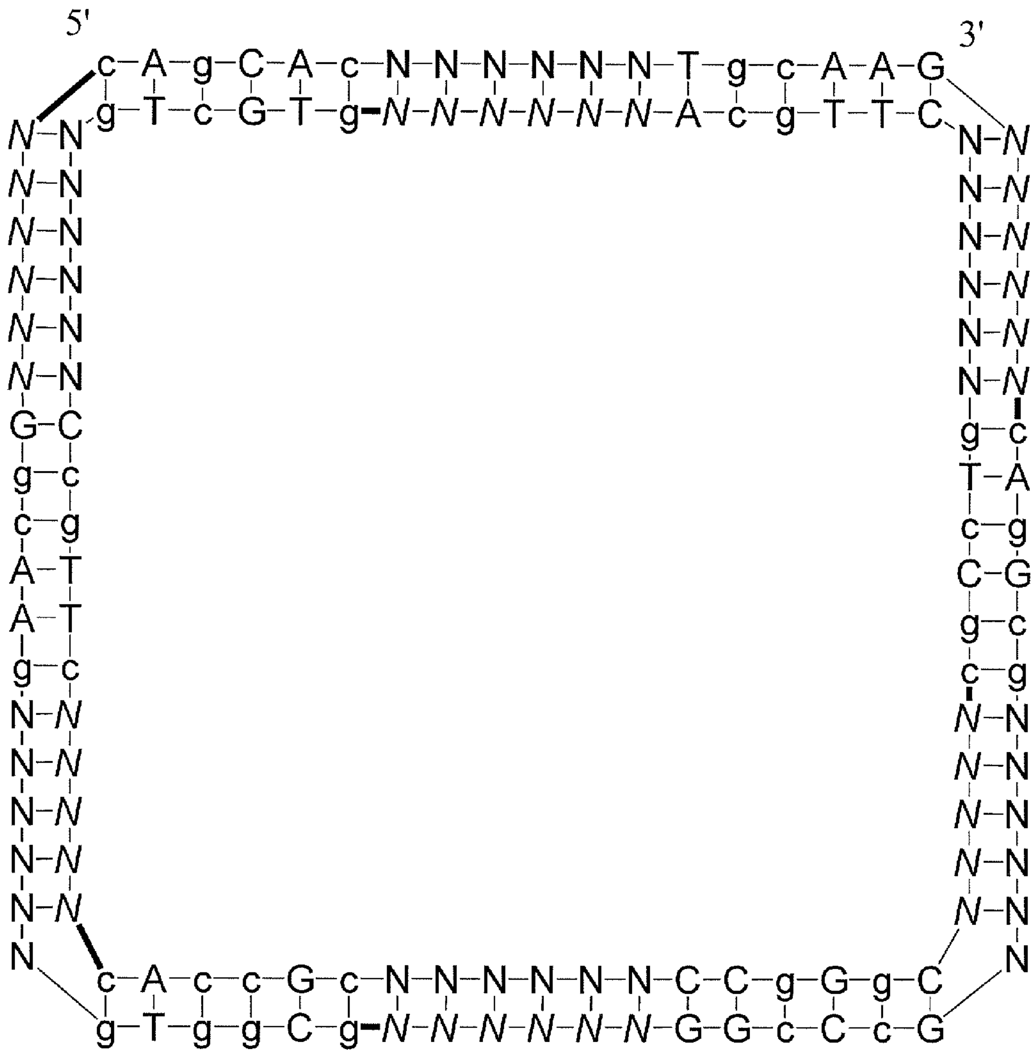


Figure 10


5'
3'
c-A-g-C-A-c-N-N-N-N-N-N-T-g-c-A-A-G
g-T-c-G-T-g-N-N-N-N-N-N-A-c-g-T-T-C
N-N
N-N
N-N
N-N
N-N
N-N
G-C
g-c
c-g
A-T
A-T
g-c
N-N
N-N
N-N
N-N
N-N
N
N-N
N-N
N-N
N-N
N-N
N-N
g-c
T-A
c-g
C-G
g-c
c-g
N-N
N-N
N-N
N-N
N-N
N
c-A-c-c-G-c-N-N-N-N-N-N-C-C-g-G-g-C
g-T-g-g-C-g-N-N-N-N-N-N-G-G-c-C-c-G

INEXPENSIVE AUTONOMOUS ASSEMBLY OF ULTRA-LARGE (UL) DNA CONSTRUCTS

CROSS REFERENCE TO RELATED APPLICATIONS

This application is a nonprovisional application that derives from, and claims priority to, U.S. nonprovisional application Ser. No. 13/936,309 filed Jul. 9, 2013 which derived from and claimed priority to provisional patent application 61/669,295, which was filed Jul. 9, 2012, for "Inexpensive Autonomous Assembly of Ultra-Large (UL) DNA Constructs".

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

This invention was made with government support under a contract awarded by the United States Defense Advanced Research Project Agency (HR0011-12-C-0064). The government has certain rights in the invention.

THE NAMES OF THE PARTIES TO A JOINT RESEARCH AGREEMENT

Not applicable

INCORPORATION-BY-REFERENCE OF MATERIAL SUBMITTED ON A COMPACT DISC

None

BACKGROUND OF THE INVENTION

(1) Field of the Invention

This invention is in the field of nucleic acid chemistry, more specifically the synthesis of nucleic acids, more specifically to the synthesis of large DNA molecules, and more specifically to processes and procedures that create large (more than 1000 base pairs) double-stranded DNA via the assembly of large numbers of short single stranded DNA molecules having preselected sequences.

(2) Description of Related Art

Synthetic biology needs processes to enable low-cost and rapid assembly of many synthetic DNA fragments into large DNA assemblies. For example, in 2012, DARPA issued a small business grant solicitation seeking technology to assemble single-stranded synthetic fragments to give 20,000 bp ML-DNA constructs. A short while earlier, the Army Research Office issued a small business grant solicitation seeking companies to design software to allow 30,000 base pairs of single stranded DNA self-assemble to form nanostructures.

Unfortunately, the realities behind the biophysics of DNA make these goals fanciful, if the attempt is made with standard DNA. With just four nucleotides, the information density of standard DNA is too low to allow (without exquisite design) more than ca. a dozen single strands to self-assemble upon simple mixing. With more fragments containing only natural nucleotides, the vagaries of "strong" and "weak" G:C and A:T pairs, hairpins, off-target Watson-Crick hybridization, and non-Watson Crick interactions (e.g wobble and major groove binding) defeat self-assembly. These can be illustrated by mentioning the following problems:

Problem (A).

Different DNA base pairs do not contribute uniformly to duplex stability. The largest source of this non-uniformity in strand hybridization is a feature of standard DNA that joins A:T pairs by just two hydrogen bonds and G:C pairs by three. Thus, A:T pairs contribute to duplex stability consistently less than G:C pairs. This makes it challenging to design DNA fragments with different nucleotide compositions that hybridize to their complements with the same affinity.

Problem (B).

DNA strands can interact in ways outside of those specified by the canonical Watson-Crick pair. In addition to wobble pairing (e.g. G:T pairs), DNA can form major groove interactions (e.g. G-quartets). These, illustrated in FIG. **1** and FIG. **2**, can (in appropriate contexts) be stronger than Watson-Crick pairing and can defeat pairing between large numbers of single stranded DNA molecules designed solely by applying Watson-Crick rules.

Problem (C).

Intra-strand folding can defeat desired inter-strand interactions needed for hybridization, primer extension, and ligation. Hairpin structures formed by a single strand, for example, can easily disrupt inter-strand hybridization that intended for a multi-strand assembly (FIG. **3**). The easy accessibility of hairpins can be illustrated by some simple mathematics. The 5'-nucleotide of a standard DNA molecule must be G, A, T, or C. Whatever it is, it can find a complementary C, T, A, or G (respectively) with a one-in-four probability at each base farther into the sequence. Within a random sequence 64 nucleotides in length, the final one, two, and three nucleotides will find perfect complements 16 times, 4 times, and once within that sequence, on average. These will form hairpins with stems that are joined by one, two, and three perfect base pairs respectively. Stems with four or five pairs and loops of 2-5 nucleotides are adequate to disrupt hybridization. Therefore, loops must be avoided by design, and this design becomes difficult to manage as the number of fragments increase.

Problem (D):

Even if DNA had access only to Watson-Crickery, even if all nucleobase pairs contributed equally to duplex stability, and even if single strands never folded by themselves, the autonomous self-assembly problem would still not be trivial. With only four nucleotide letters to encode information, the information density of natural DNA is low. For a bacterial sized genome having a random sequence, all 10mers are present once. Overlapping 10mers are more than adequate to support ligation, even if they include one or two mismatches, at temperatures when typical ligases operate. This low information density makes it essentially impossible to do reliable self-assembly from any more than a dozen or so fragments. Each complement is present at low concentrations, making the rates at which they find each other low a priori. The rate of hybridization is slowed as GACT DNA fragments find "off target" GACT fragments, bind to them, and dwell for a time before dissociating to seek their "on target" fragments.

Given these realities of the chemical structure of natural DNA, it is hardly surprising that Nature rarely does what synthetic biologists want to do: Large-scale assembly by way of the hybridization of multiple single stranded fragments. Non-uniformity in the binding of sequences of natural nucleotides make it essentially impossible to assemble by autonomous hybridization of thousands (or more) nucleobase pairs, even if the primary products have no errors at all. Therefore, in natural biology, large-scale DNA assemblies are carried forward carefully from generation to generation, with strand displacement at the core of polymerization and specifically targeted ligation events that do not allow the DNA to wander into multiple single strands.

Thus, most large synthetic DNA (LS-DNA) molecules today are obtained via the "Gibson method" [Gibson 2011], rather than the spontaneous self-assembly of many single DNA strands prepared by synthesis. The Gibson method reproduces in vitro the natural Szostak process for recombination in vivo [Szostak et al. 1983]. It starts with pre-annealed duplexes, cuts them back with a 3'-exonuclease to generate sticky ends (without cutting back so far as to disrupt the duplex) and then uses the resulting sticky ends to assemble the duplexes with overhangs. Expert intervention is required at many steps in the process, creating costs.

While [Gibson 2011] speaks of single strand assembly, including single stranded assembly in yeast cells [Gibson 2009], they teach that to "ensure that error-free molecules are obtained at a reasonable efficiency, only eight to twelve 60-base oligos are assembled at one time" [Gibson 2011]. This teaching, we presume, reflects the problems listed above, which are deeply embedded in the molecular structure of natural DNA. These drive the need for inventive processes to allow LS-DNA assembly from multiple single stranded synthetic DNA fragments.

BRIEF SUMMARY OF THE INVENTION

This invention provides processes to assemble more than 12, and preferably at least 20, strands of single stranded DNA fragments, preferably 50-100 nucleotides in length, and more preferably 50-80 nucleotides in length, to give large synthetic DNA (LS-DNA) constructs. Those fragments intended to be ligated must have free 5'-phosphate groups at the ends to be ligated and/or free 3'-hydroxyl groups at the ends to be ligated. Those fragments intended to be extended must have free 3'-hydroxyl groups at the ends to be extended. Further, only those 3'-ends annealed to another of the DNA fragments with a 3'-underhang will be extended (FIGS. **5**-**10**). As useful applications, this invention therefore allows the assembly, from a collection of single stranded DNA fragments, of genes encoding whole proteins (typically 1000-3000 nucleotide pairs), plasmid-sized constructs (5000-10000 base pairs), artificial chromosomes (such as BACs having 10,000 to 300,000 nucleotide pairs), or even chromosomes (having a million or more nucleotide pairs).

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWING(S)

FIG. **1**. Illustration of Problem (A). Wobble pairing can cause single stranded oligonucleotides to not interact as predicted by Watson-Crick rules, especially in an annealing attempt that involves more than 12 single stranded oligonucleotides (and certainly 20 or more). As AEGIS pairs are all joined by three hydrogen bonds, AEGIS pairs are stronger than A:T pairs, allowing correct pairs to dominate over wobble, which involve two hydrogen bonds [Benner et al. 2010].

FIG. **2**. Further illustration of Problem (A). Major groove binding interactions can cause single stranded oligonucleotides to not anneal as predicted by Watson-Crick pairing, especially if the annealing attempt involves more than 12 single stranded oligonucleotides (and certainly 20 or more). Various of the AEGIS purines cannot form these major groove interactions, if they are implemented in a form that lacks a nitrogen atom at what would be formally called "position 7" on the analogous purine. These include P and 7-deazaisoguanosine.

FIG. **3**. Illustration of Problem (B). Hairpins prevent certain single stranded oligonucleotides from interacting with their target as predicted by Watson-Crick pairing. Hairpins that involving a folding back of the 3'-end also can be extended by polymerases to give undesired products. While hairpins can be avoided by careful design if only a few oligonucleotides are being assembled, they are difficult to avoid by design if the assembly attempt involves more than 12 single stranded oligonucleotides (and certainly for 20 or more). Hairpins cannot form if the ends contain AEGIS components (shown in cartoon form).

FIG. **4**. Watson-Crick pairing follow two complementarity rules: (a) size (large purines pair with small pyrimidines) and (b) hydrogen bonding (on purine pu and pyrimidine py ring analogs, hydrogen bond acceptors, A, pair with donors D). Rearranging D and A groups on the nucleobases creates artificially expanded genetic information systems (AEGIS). A central teaching of this application is that various heterocyclic systems can implement the same hydrogen bonding interaction. For example, isoguanine and 7-deazaisoguanine both implement the puDDA hydrogen bonding pattern.

FIG. **5**. (a) Proposed chemical mechanisms for the overall conversion of Z:P pairs to C:G pairs, completed with two copying steps. (b) Copying steps are shown, where the first polymerase extension cycle creates a natural strand by placing C opposite P and G opposite B. Alternatively, if dZTP or dPTP is present in small amounts, the conversion can be completed in three or more cycles, or in the products of PCR amplification.

FIG. **6**. (a) Proposed chemical mechanisms for the overall conversion of S:B pairs to T:A pairs, completed with three copying steps, where the minor enol tautomer of isoG pairs with T. (b) Copying steps shown involve three cycles, where a small amount of dBTP is incorporated to allow the dS in the ligated product to first be copied with a dB.

FIG. **7**. Cartoon illustrating the annealing and extension parts of the anneal-extend-ligate process using AEGIS components and a hypothetical cyclic target. The AEGIS components shown are Z and P; analogous cartoons can be drawn using the AEGIS components S and B. The DNA strands are preselected to have regions that "partially overlap", meaning that the 3'-end of one or more of the fragments anneals to another fragment so that its 3'-end can be extended using its annealed partner as a template. The overlapping annealed segments preferably have two or more AEGIS components, although a single AEGIS component is not excluded. The extension must be done by a polymerase that is not "strand displacing"; an example is Phusion DNA polymerase. Shown are not actual sequences, but rather generic sequences to illustrate the concept; therefore sequence listing entries are not required by statute, regulations, or USPTO procedures. In practice, the paired regions are longer (presently preferred 12-20 nucleotides, more preferably 15-18, with similar melting temperatures), and the single stranded regions to be copied are longer. The presently preferred total length of the fragments is 40-100 nucleotides, more preferably 50-80 nucleotides, and most preferably 50-60 nucleotides, with the preferred length dependent on the level of error in the synthetic DNA fragments themselves.

FIG. **8**. Cartoon illustrating the extended products ready for the ligation part of the anneal-extend-ligate process using AEGIS components and a hypothetical cyclic target. N's in italics are those added by the polymerase against the N's in the preselected sequence. These are not actual sequences, but rather generic sequences to illustrate the concept; therefore sequence listing entries are not required by statute, regulations, or USPTO procedures. In practice, the paired regions are longer (presently preferred 12-20 nucleotides, more preferably 15-18, with similar melting temperatures), and the single stranded regions to be copied are longer. The presently preferred total length of the fragments is 40-100 nucleotides, more preferably 50-80 nucleotides, and most preferably 50-60 nucleotides, with the preferred length dependent on the level of error in the synthetic DNA fragments themselves.

FIG. **9**. Cartoon illustrating the product after ligation of the anneal-extend-ligate process using AEGIS components and a hypothetical cyclic target. These are not actual sequences, but rather generic sequences to illustrate the concept; therefore sequence listing entries are not required by statute, regulations, or USPTO procedures. In practice, the paired regions are longer (presently preferred 12-20 nucleotides, more preferably 15-18, with similar melting temperatures), and the single stranded regions to be copied are longer. The presently preferred total length of the fragments is 40-100 nucleotides, more preferably 50-80 nucleotides, and most preferably 50-60 nucleotides, with the preferred length dependent on the level of error in the synthetic DNA fragments themselves.

FIG. **10**. Cartoon illustrating the conversion step following the anneal-extend-ligate process using AEGIS components and a hypothetical cyclic target. Lower case nucleotides are those that arose from conversion. These are not actual sequences, but rather generic sequences to illustrate the concept; therefore sequence listing entries are not required by statute, regulations, or USPTO procedures. This is illustrated with Z:P conversion; see Example 1 for S:B conversion. In practice, the paired regions are longer (presently preferred 12-20 nucleotides, more preferably 15-18, with similar melting temperatures), and the single stranded regions to be copied are longer. The presently preferred total length of the fragments are 40-100 nucleotides, more preferably 50-80 nucleotides, and most preferably 50-60 nucleotides, with the preferred length dependent on the level of error in the synthetic DNA fragments themselves.

DETAILED DESCRIPTION OF THE INVENTION

(1) Use of Non-Standard Nucleotides to Address the Problems in Multi-Fragment Assembly

The instant invention add nucleotides to the "alphabet" of standard DNA, specifically, components of an artificially expanded genetic information systems (AEGIS) (FIG. **4**). By shuffling hydrogen bond donor and acceptor groups, AEGIS adds up to eight nucleotides to the four (G, A, T and C) found in standard DNA. These form four orthogonal additional pairs between AEGIS complements that allow AEGIS DNA to bind to complementary DNA but not to standard GACT DNA [Benner et al. 2010] joined by "non-standard hydrogen bonding patterns" These are illustrated in FIG. **4**, with the hydrogen bonding pattern defined by the prefix "py" (to indicate a heterocycle with a single six-membered ring) or "pu" (to indicate a heterocycle with a single six membered ring fused to a single five-membered ring) followed by "donor" or "acceptor", proceeding from the major groove to the minor groove. The pattern is "non standard" if it differs from the patterns of hydrogen bonding groups found in G, A, aminoA, C, T, or U, or other heterocycles that implement the same hydrogen bonding pattern, regardless of the choice of the heterocycle upon which to implement that hydrogen bonding pattern. A teaching of this specification is that the same hydrogen bonding pattern can be implemented by more than one heterocycle.

AEGIS pairs have been designed to not suffer from many of the problems found in natural DNA. First, all nucleobase pairs are joined by three hydrogen bonds (FIG. **4**), addressing Problem (A) in natural DNA. This means that AEGIS nucleobase pairs are not sometimes strong and sometimes weak (the standard C:G pair is strong; the T:A pair is weak), but rather are uniformly strong.

Second, the AEGIS nucleobases are designed so as to not have Hoogsteen and other major groove non-canonical hydrogen bonding possibilities (FIG. **2**). Thus, in regions where AEGIS nucleotides are incorporated, Problem (B) cannot confound desired hybridization.

Third, adding nucleotide letters to an expanded genetic alphabet increases the information density of the resulting DNA sequences, addressing Problem (D). With six or eight different nucleotide letters, and certainly with 10 or 12, even a mixture of 10,000 fragments, each 100 nucleotides long, does not have close "off-target" mismatches that can, kinetically, slow the rate of hybridization or, thermodynamically, generate undesired hybrids that compete with the formation of desired hybrids.

The added information density can be used strategically to solve Problem (C). For example, if a segment of DNA is built entirely from standard GACT nucleotides, and if AEGIS nucleotides are at the ends where ligation will take place, it is impossible for hairpin structures to form, and therefore impossible for hairpin structures to compete with desired ligation (FIG. **3**).

As reduction to practice, the oligonucleotides built from AEGIS components are prepared by standard phosphoramidite-based phosphoramidite synthesis from phosphoramidites [Yang et al 2011, and references cited therein]. These procedures are described in the following publications and patents, and publications that these cite, all incorporated in their entirety by reference.

(2) Optional Conversion

Of course, any construct that has self-assembled autonomously around AEGIS fragments will not at this point be completely natural DNA. It will contain unnatural AEGIS nucleotides embedded throughout the LS-DNA construct.

This unnaturalness need not necessarily limit the application of such constructs should bacteria become available that accept AEGIS DNA. Nor will it be an issue should the constructs be used to support nanostructures that need not enter natural biological systems downstream.

However, many synthetic biologists want entirely natural LS-DNA constructs. Therefore, the process of the invention can optionally include a step that converts AEGIS pairs to give standard pairs. This conversion exploits the recipes disclosed in U.S. patent application Ser. No. 12/653,613, which is incorporated in its entirety herein by reference. While not wishing to be bound by theory, the key to this conversion is the ability of a polymerase, if it does not have available a complementary non-standard nucleoside triphosphate, to create the best Watson-Crick mismatch between a non-standard AEGIS component in a template and a standard nucleotide, based on (for example) alternative protonation/deprotonation states and/or alternative tautomeric forms.

Conversion in its various embodiments is exemplified by two examples, one involving the dZ:dP pair, and the other involving the dS:dB AEGIS pair. While not wishing to be bound by theory, protonation of the dC:dP mismatch allows the misincorporation of dC by a polymerase opposite dP in a template (FIG. **5**) in the absence of a complement to the template dP. Deprotonation of the dG:dZ mismatch allows the misincorporation of dG by a polymerase opposite dZ in a template, again in absence of dPTP. In the next cycle of copying, the newly incorporated C then directs the incorporation of dG, finishing the conversion of dP:dZ pairs to dG:dC pairs.

Polymerases can, again according to theory, use this deprotonated pair to direct the misincorporation of dG opposite dZ in a template In the next cycle of copying, the newly incorporated dG then directs the incorporation of dC, finishing the conversion of dZ:dP pairs to dC:dG pairs. This can be done in two or three steps, three steps if small amounts of dZTP and/or dPTP are added.

While not wishing to be bound by theory, for dS and dB, a minor tautomeric form of the puDDA hydrogen bonding pattern, when implemented using the isoguanine heterocycle, supports a mismatch with thymine (FIG. **6**). Polymerases can, again according to theory, use this minor tautomer to direct the misincorporation of dT opposite dB in a template in the absence of a complement to the major tautomer of isoguanine. In the next cycle of copying, the newly incorporated dT then directs the incorporation of dA, finishing the conversion of dS:dB pairs to dT:dA pairs.

Since dS does not have an analogous mechanism to support an dS:dA mismatch, the presently preferred conversion process includes a small amount of disoGTP, to match to disoC in the template. The newly incorporated isoguanine then, in its minor tautomeric form, is mismatched against thymine, and the overall conversion of these dS:dB pairs to dT:dA pairs is completed with three copying steps.

Conversion can also be effected with RNA assemblies involving reverse transcriptase, and other implementations of the AEGIS non-standard hydrogen bonding schemes.

Thus, after autonomous self-assembly of independently synthesized fragments using the orthogonality of AEGIS nucleotides, the AEGIS nucleotides are removed to give an entirely natural full-length DNA product. Of course, the synthetic fragments must be designed so as to have Z present at sites where C is desired in the final LS-DNA product, P is present at sites where G is desired in the final LS-DNA product, S is present at sites where T is desired in the final LS-DNA product, and B is present at sites where A is desired in the final LS-DNA product. Additional rules can be specified depending on the nature of the conversion process.

(3) References

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(4) Examples

Synthesis of Preselected DNA Oligonucleotide Fragments

The invention was demonstrated by the assembly of a gene that encodes a protein that confers resistance on kanamycin. The following fragments were synthesized on solid phase.

```
                                        SEQ ID NO 01
CACCATGAGCCATATTCAACGGGAAACGTCGAGGCCGCGATTAAATTCCAA
CATGGASGCSGASTT

                                        SEQ ID NO 02
SGASTGCCCGACBTTATCGCGAGCCCATTTATACCCATATAABTCBGCBTC
C

                                        SEQ ID NO 03
SGTCGGGCABTCBGGTGCGACAATCTATCGCTTGTATGGGAAGCCCGASGC
GCCBGAG

                                        SEQ ID NO 04
BACBTCBTTGGCBACGCTACCTTTGCCATGTTTCAGAAACAACTCSGGCGC
BT

                                        SEQ ID NO 05
SGCCAASGASGTSACAGATGAGATGGTCAGACTAAACTGGCTGACGGABTT
TATGCCSCTSC

                                        SEQ ID NO 06
TCBTCBGGBGTBCGGATAAAATGCTTGATGGTCGGBAGBGGCATAAAST

                                        SEQ ID NO 07
SACSCCSGASGATGCATGGTTACTCACCACTGCGATCCCCGGBAAAACBGC
BTT

                                        SEQ ID NO 08
CBACAATBTTSTCBCCTGAATCAGGATATTCTTCTAATACCTGGAASGCSG
TTTTSC

                                        SEQ ID NO 09
SGABAASATTGTSGATGCGCTGGCAGTGTTCCTGCGCCGGTTGCASTCGAT
TCCTGTST

                                        SEQ ID NO 10
GBGCGAGBCGAAATACGCGATCGCTGTTAAAAGGACAATTACABACAGGAA
TCGABT

                                        SEQ ID NO 11
TCGSCTCGCSCAGGCGCAATCACGAATGAATAACGGSTTGGTSGASG

                                        SEQ ID NO 12
CSTGSTCBACBGGCCAGCCATTACGCTCGTCATCAAAATCACTCGCBTCBA
CCAABC

                                        SEQ ID NO 13
SGTSGABCABGTCTGGAAAGAAATGCASAABCTSTTGCCBT

                                        SEQ ID NO 14
BTCBAGSGAGAABTCACCATGAGTGACGACTGAATCCGGTGAGAASGGCAA
BAGSTTBT

                                        SEQ ID NO 15
ASTTCTCBCTSGASAACCTTATTTTTGACGAGGGGAAATTAATAGGTTGTA
TTGASGTTGGACGBGT
```

-continued

SEQ ID NO 16
CBAGBTCCTGGTASCGGTCTGCGATTCCGACSCGTCCAACBTCA

SEQ ID NO 17
BTACCAGGASCTSGCCATCCTATGGAACTGCCTCGGTGAGTTTTCTCCSTC

BTTACAGAABC

SEQ ID NO 18
STTBTTCATBTCBGGATTATCAATACCATATTTTTGAAAAAGCCGSTTCTG

TAASGABG

SEQ ID NO 19
CSGASATGAASAABTTGCAGTTTCATTTGATGCTCGATGAGTTTTTCTAAC

AGGATCCGCBCGBCSAG

SEQ ID NO 20
CTAGSGGSCGBTCSGTCCGTCCTGTCAGCTGCTBGSCGSGCG

The sequence for the protein that encodes kanamycin resistance was obtained from the literature. Using a software tool designed for this purpose, a gene that encodes the protein was broken down to 20 single stranded oligonucleotide fragments containing S (5-methyl-2'-deoxyisocytidine) and B (2'-deoxyisoguanosine) nucleotides at their 5' and 3' ends, ranging in size from 41 to 67 nucleotides (nts) (Table 1). These were the DNA oligonucleotide "fragments" with pre-selected sequences that, with partial overlap, would self-assemble in hybridization including S:B pairs, as the first step in the anneal-extend-ligate process.

Additionally, the LS-DNA product was designed to have a CACC tetranucleotide immediately upstream from the start codon; this assisted cloning into a TOPO expression vector. It was also designed to have a Bam HI region downstream of the stop codon. The complete designed sequence that is the intended product from the process of the instant invention was therefore 863 nts (Table 2).

Annealing, Extending and Ligating the Preselected DNA Oligonucleotide Fragments

The "anneal-extend-ligate" procedure was then executed with these 20 fragments, prepared by solid phase phosphoramidite synthesis. The procedure followed two different methods (AEL #1 and AEL #2). In each case, DNA (125-250 ng or each fragment) was used. Working stocks of 10 μM dNTPs were mixed with 5×ISO buffer and water to a final volume of 40 μL (one μL each oligo, 8 μL 5×ISO buffer, 12 μL water) in duplicate, and heated with the fragments, and then slowly cooled. Incubation programs were:

AEL #1: 95° C. for 5 minutes, temperature reduced at 0.1° C./second to 45° C.

AEL #2: 75° C. for 20 minutes, temperature reduced at 0.1° C./second to 60° C., held 30 minutes, temperature reduced 0.1° C./second to 4° C.

Aliquots (5 μL) of each annealing mixture were transferred to a new tube to which was added enzyme mixture containing polymerases, ligases, and substrates (15 μL). The mixtures were then incubated at 48° C. for 60 minutes to permit primer extension (using a non-strand displacing polymerase active at this temperature, preferably Phusion) in a procedure resembling that of [Gibson 2011]. The samples were then cooled and stored at 4° C. until PCR amplified.

PCR Amplification with Conversion of the Ligated LS-DNA Product

PCR reactions set up with 1 μL assembly mixture and either no added disoGTP (dBTP) or 0.5 μL added dBTP. The forward and reverse primers were:

KanR For: CACCATGAGCCATATTCAACGG SEQ ID NO 21

KanR Rev: GTCCGTCCTGTCAGCTGC SEQ ID NO 22

As the reverse primer was designed to be upstream of the final AEGIS nucleotides, the final PCR product was 849 bp. The PCR cycling program was: 95° C. 2 minutes, followed by 30 cycles of 95° C. 40 seconds, 55° C. 20 seconds, 72° C. 2 minutes, with a final extension of 72° C. 15 minutes.

TABLE 2

PCR Recipe

Item	Per rxn	MMix (×6)
Taq Buffer, 10X	5 μL	30 μL
dNTP (10 mM)	1 μL	6 μL
KanR For (10 μM)	2 μL	12 μL
KanR Rev (10 μM)	2 μL	12 μL
Taq polymerase	0.4 μL	2.4 μL
DNA or water	1 μL	—
Water	38.6 μL	231.6 μL
dBTP (10 mM)	0.3 or 0 μL	—

Two methods were used for the PCR amplification (with conversion) of the annealed, extended, and ligated construct. In the second, 2'-deoxyisoguanosine triphosphate (dBTP) is present in small amounts; in the first, it is absent. Gel electrophoretic analysis of the products showed the largest at the expected length of 863 bp. Ladder bands were observed, and presumed to represent incomplete assemblies. The same ladder structure is evident for both incubation methods, and both methods resulted in strong doublet products as the top of the gel.

The second method (where dBTP is present) appears to give more of the desired product, and is presently preferred. While not wishing to be bound by theory, we interpret this result as evidence that the presence of dBTP facilitates the copying of templates containing dS.

The PCR product was then ligated into a TOPO expression vector. This was used to transform Top10 cells by electroporation. Transformed cells were grown in the presence of kanamycin, harvested, and the plasmid recovered, and a sampling of the recovered plasmids was sequenced. The sequences (Table 2) showed essentially no error in the annealing, extension, ligation, or conversion steps; they did show errors common in sequences at the ends of long reads.

To show that the LS-DNA was functional, *E. coli* cells containing it were plated on LB/agar containing kanamycin (100 μg/mL) and IPTG (0.1 mm) from cultures prepared from BL21(DE3) cells transformed with pET TOPO expression vector containing the insert encoding for the kanamycin resistance protein. A negative control plate was spread with a culture of cells containing vector but no insert, a fact confirmed with PCR using vector primers.

TABLE 2

5 Sequences of gene for kanamycin resistance. AEGIS bases are highlighted

KanR_AEGIS	CTAGTGGSCGBTCTGSCCGTCCTGTCAGCTGCTBGSCGSGCGGATCCTGT	SEQ ID NO 23
KanR_normal	---T	SEQ ID NO 24
Kan09 with dBTP	--------------GTCCGACCTGTCAGCTGCTAGTCGTGCGGATCCTGT	SEQ ID NO 25

TABLE 2-continued

5 Sequences of gene for kanamycin resistance. AEGIS bases are highlighted

Kan11 with dBTP	--------------GTCCGTCCTGTCAGCTGCTAGTCGTGCGGATCCCGT	SEQ ID NO 26
Kan14 with dBTP	------------------GTCCTGCCAGCTGCTATTCGGGGGGATCCTGT	SEQ ID NO 27
Kan12 without dBTP	--------------GTCCGTCCTGTCAGCTGCTAGTCGTGCGGATCCTGT	SEQ ID NO 28
Kan13 without dBTP	--------------GTCCGTCCTGTCAGCTGCTAGTCGTGCGGATCCTGT	SEQ ID NO 29
KanR_AEGIS	TAGAAAAACTCATCGAGCATCAAATGAAACTGCAASTTBTTCATBTCBGG	SEQ ID NO 23
KanR_normal	TAGAAAAACTCATCGAGCATCAAATGAAACTGCAATTTATTCATATCAGG	SEQ ID NO 24
Kan09 with dBTP	CAGAAAAACTCATCGAGCATCAAATGAAACTGCAATTTGTTCATATCCGG	SEQ ID NO 25
Kan11 with dBTP	TAGAAAAACTCATCGAGCATCAAATGAAACTGCAATTTATTCATATCAGG	SEQ ID NO 26
Kan14 with dBTP	TAGATCC-TTCATCGAGCATCATATGAAACTGCAATTTATTCATATCAGG	SEQ ID NO 27
Kan12 without dBTP	TAGAAAAACTCATCGAGCATCAAATGAAACTGCAATTTATTCATATCAGG	SEQ ID NO 28
Kan13 without dBTP	TAGAAAAACTCATCGAGCATCAAATGAAACTGCAATTTATTCATATCAGG	SEQ ID NO 29
KanR_AEGIS	ATTATCAATACCATATTTTTGAAAAAGCCGSTTCTGTAASGABGGAGAAA	SEQ ID NO 23
KanR_normal	ATTATCAATACCATATTTTTGAAAAAGCCGTTTCTGTAATGAAGGAGAAA	SEQ ID NO 24
Kan09 with dBTP	ATTATCAATACCATATTTTTGAAAAAGCCGTTTCTGTAATGAAGGACAAA	SEQ ID NO 25
Kan11 with dBTP	ATTATCAATACCATATTTTTGAAAAAGCCGTTTCTGTAATGAAGGAGAAA	SEQ ID NO 26
Kan14 with dBTP	ATTATCAATACCATATTTTTGAAAAAGCTTTTTCTGCAATGACCGAAAAA	SEQ ID NO 27
Kan12 without dBTP	ATTATCAATACCATATTTTTGAAAAAGCCGTTTCTGTAATGAAGGAGAAA	SEQ ID NO 28
Kan13 without dBTP	ATTATCAATACCATATTTTTGAAAAAGCCGCTTCTGTAATGAAGGAGAAA	SEQ ID NO 29
KanR_AEGIS	ACTCACCGAGGCAGTTCCATAGGATGGCBAGBTCCTGGTASCGGTCTGCG	SEQ ID NO 23
KanR_normal	ACTCACCGAGGCAGTTCCATAGGATGGCAAGATCCTGGTATCGGTCTGCG	SEQ ID NO 24
Kan09 with dBTP	ACTCACCGAGGCAGTTCCATAGGATGGCAAGATCCTGGTATCGGTCTGCG	SEQ ID NO 25
Kan11 with dBTP	ACTCACCGAGGCAGTTCCATAGGATGGCAAGATCCTGGTATCGGTCTGCG	SEQ ID NO 26
Kan14 with dBTP	ACTCACCGAGGCAGTTCCATAGGATGGCAAGATCCTGGTATCGGTCTGCG	SEQ ID NO 27
Kan12 without dBTP	ACTCACCGAGGCAGTTCCATAGGATGGCAAGATCCTGGTATCGGTCTGCG	SEQ ID NO 28
Kan13 without dBTP	ACTCACCGAGGCAGTTCCATAGGATGGCAAGATCCTGGTATCGGTCTGCG	SEQ ID NO 29
KanR_AEGIS	ATTCCGACSCGSCCBACBTCAATACAACCTATTAATTTCCCCTCGTCAAA	SEQ ID NO 23
KanR_normal	ATTCCGACTCGTCCAACATCAATACAACCTATTAATTTCCCCTCGTCAAA	SEQ ID NO 24
Kan09 with dBTP	ATTCCGACTCGTCCAACATCAATACAACCTATTAATTTCCCCTCGTCAAA	SEQ ID NO 25
Kan11 with dBTP	ATTCCGACTCGTCCAACATCAATACAACCTATTAATTTCCCCTCGTCAAA	SEQ ID NO 26
Kan14 with dBTP	ATTCCGACTCGTCCAACATCAATACAACCTATTA-TTTCCCCTCGTCAAA	SEQ ID NO 27
Kan12 without dBTP	ATTCCGACTCGTCCAACATCAATACAACCTATTAATTTCCCCTCGTCAAA	SEQ ID NO 28
Kan13 without dBTP	ATTCCGACTCGTCCAACATCAATACAACCTATTAATTTCCCCTCGTCAAA	SEQ ID NO 29
KanR_AEGIS	AATAAGGTTBTCBAGSGAGAABTCACCATGAGTGACGACTGAATCCGGTG	SEQ ID NO 23
KanR_normal	AATAAGGTTATCAAGTGAGAAATCACCATGAGTGACGACTGAATCCGGTG	SEQ ID NO 24
Kan09 with dBTP	AATAAGGTTATCAAGTGAGAAATCACCATGAGTGACGACTGAATCCCGTG	SEQ ID NO 25
Kan11 with dBTP	AATAAGGTTATCAAGTGAGAAATCACCATGAGTGACGACTGAATCCGGTG	SEQ ID NO 26
Kan14 with dBTP	AATAAGGTTATCAAGAGAGAAATCTCCATGAGTGACGACTGAATTTTGTA	SEQ ID NO 27
Kan12 without dBTP	AATAAGGTTATCAAGTGAGAAATCACCATGAGTGACGACTGAATCCGGTG	SEQ ID NO 28
Kan13 without dBTP	AATAAGGTTATCAAGTGAGAAATCACCATGAGTGACGACTGAATCCGGTG	SEQ ID NO 29
KanR_AEGIS	AGAASGGCAABAGSTTBTGCATTTCTTTCCAGACSTGSTCBACBGGCCAG	SEQ ID NO 23
KanR_normal	AGAATGGCAAAAGTTTATGCATTTCTTTCCAGACTTGTTCAACAGGCCAG	SEQ ID NO 24
Kan09 with dBTP	AGAATGGCAAAAGTTTATGCATTTCTTTCCAGACTTGTTCAACAGGCCAG	SEQ ID NO 25
Kan11 with dBTP	AGAATGGCAAAAGTTTATGCATTTCTTTCCAGACTTGTTCAACAGGCCAG	SEQ ID NO 26
Kan14 with dBTP	AGAATGGCAAAAGTTTATGCATTTCTTTCCAGACTTGATCAACAGGCCAG	SEQ ID NO 27
Kan12 without dBTP	AGAATGGCAAAAGTTTATGCATTTCTTTCCAGACTTGTTCAACAGGCCAG	SEQ ID NO 28
Kan13 without dBTP	AGAATGGCAAAAGTTTATGCATTTCTTTCCAGACTTGTTCAACAGGCCAG	SEQ ID NO 29
KanR_AEGIS	CCATTACGCTCGTCATCAAAATCACTCGCBTCBACCAABCCGTTATTCAT	SEQ ID NO 23
KanR_normal	CCATTACGCTCGTCATCAAAATCACTCGCATCAACCAAACCGTTATTCAT	SEQ ID NO 24
Kan09 with dBTP	CCATTACGCTCGTCATCAAAATCACTCGCATCAACCAACCCGTTATTCAT	SEQ ID NO 25
Kan11 with dBTP	CCATTACGCTCGTCATCAAAATCACTCGCATCAACCAAACCGTTATTCAT	SEQ ID NO 26
Kan14 with dBTP	CCATTACGOTCGTCATCAAAATCACTCGCATCAACCAACCCGTTATTCAT	SEQ ID NO 27
Kan12 without dBTP	CCATTACGCTCGTCATCAAAATCACTCGCATCAACCAAACCGTTATTCAT	SEQ ID NO 28
Kan13 without dBTP	CCATTACGCTCGTCATCAAAATCACTCGCATCCACCAAACCGTTATTCAT	SEQ ID NO 29
KanR_AEGIS	TCGTGATTGCGCCTGBGCGAGBCGAAATACGCGATCGCTGTTAAAAGGAC	SEQ ID NO 23
KanR_normal	TCGTGATTGCGCCTGAGCGAGACGAAATACGCGATCGCTGTTAAAAGGAC	SEQ ID NO 24
Kan09 with dBTP	TCGTGATTGCGCCTGAGCGAGACGAAATACGCGATCGCTGTTAAAAGGAC	SEQ ID NO 25
Kan11 with dBTP	TCGTGATTGCGCCCGAGCGAGACGAAATACGCGATCGCTGTTAAAAGGAC	SEQ ID NO 26
Kan14 with dBTP	TCGTGATTGCGCCTGAGCGAGACGAAATACGCGATCGCTGTTAAAAGGAC	SEQ ID NO 27
Kan12 without dBTP	TCGTGATTGCGCCTGAGCGAGACGAAATACGCGATCGCTGTTAAAAGGAC	SEQ ID NO 28
Kan13 without dBTP	TCGTGATTGCGCCTGAGCGAGACGAAATACGCGATCGCTGTTAAAAGGAC	SEQ ID NO 29
KanR_AEGIS	AATTACBAACBGGAATCGABTGCAACCGGCGCAGGAACACTGCCAGCGCA	SEQ ID NO 23
KanR_normal	AATTACAAACAGGAATCGAATGCAACCGGCGCAGGAACACTGCCAGCGCA	SEQ ID NO 24
Kan09 with dBTP	AATTACAAACAGGAATCGAATGCAACCGGCGCAGGAACACTGCCAGCGCA	SEQ ID NO 25
Kan11 with dBTP	AATTACAAACAGGAATCGAATGCAACCGGCGCAGGAACACTGCCAGCGCA	SEQ ID NO 26
Kan14 with dBTP	AATTACAAACAGGAATCGAATGCAACCGGCGCAGGAACACTGCCAGCGCA	SEQ ID NO 27
Kan12 without dBTP	AATTACAAACAGGAATCGAATGCAACCGGCGCAGGAACACTGCCAGCGCA	SEQ ID NO 28
Kan13 without dBTP	AATTACAAACAGGAATCGAATGCAACCGGCGCAGGAACACTGCCAGCGCA	SEQ ID NO 29

TABLE 2-continued

5 Sequences of gene for kanamycin resistance. AEGIS bases are highlighted

KanR_AEGIS	TCBACAATBTTSTCBCCTGAATCAGGATATTCTTCTAATACCTGGAASGC		SEQ ID NO 23
KanR_normal	TCAACAATATTTTCACCTGAATCAGGATATTCTTCTAATACCTGGAATGC		SEQ ID NO 24
Kan09 with dBTP	TCAACAATATTTTCACCTGAATCAGGATATTCTTCTAATACCTGGAATGC		SEQ ID NO 25
Kan11 with dBTP	TCAACAATATTTTCACCTGAATCAGGATATTCTTCTAATACCTGGAATGC		SEQ ID NO 26
Kan14 with dBTP	TCAACAATATTTTCACCTGAATCAGGATATTCTTCTAATACCTGGAATGC		SEQ ID NO 27
Kan12 without dBTP	TCAACAATATTTTCACCTGAATCAGGATATTCTTCTAATACCTGGAATGC		SEQ ID NO 28
Kan13 without dBTP	TCAACAATATTTTCACCTGAATCAGGATATTCTTCTAATACCTGGAATGC		SEQ ID NO 29
KanR_AEGIS	SGTSTTSCCGGGGATCGCAGTGGTGAGTAACCATGCATCBTCBGGBGTBC		SEQ ID NO 23
KanR_normal	TGTTTTTCCGGGGATCGCAGTGGTGAGTAACCATGCATCATCAGGAGTAC		SEQ ID NO 24
Kan09 with dBTP	TGTTTTTCCGGGGATCGCAGTGGTGAGTAACCATGCATCATCAGGAGTAC		SEQ ID NO 25
Kan11 with dBTP	TGTTTTTCCGGGGATCGCAGTGGTGAGTAACCATGCATCATCAGGAGTAC		SEQ ID NO 26
Kan14 with dBTP	TGTTTTTCCOGGGATCGCAGTGGTGAGTAACCATGCATCATCAGGAGTAC		SEQ ID NO 27
Kan12 without dBTP	TGTTTTTCCGGGGATCGCAGTGGTGAGTAACCATGCATCATCAGGAGTAC		SEQ ID NO 28
Kan13 without dBTP	TGTTTTTCCGGGGATCGCAGTGGTGAGTAACCATGCATCATCAGGAGTAC		SEQ ID NO 29
KanR_AEGIS	GGATAAAATGCTTGATGGTCGGBAGBGGCATAAA-STCCGTCAGCCAGTT		SEQ ID NO 23
KanR_normal	GGATAAAATGCTTGATGGTCGGAAGAGGCATAAA-TTCCGTCAGCCAGTT		SEQ ID NO 24
Kan09 with dBTP	GGATAAAATGCTTGATGGTCGGAAGAGGCATAAA-TTCCGTCAGCCAGTT		SEQ ID NO 25
Kan11 with dBTP	GGATAAAATGCTTGATGGTCGGAAGAGGCATAAA-TTCCGTCAGCCAGTT		SEQ ID NO 26
Kan14 with dETP	GGATAAAATGCTTGATGGTCGGAAGAGGCATAAA-TTCCGTCAGCCAGTT		SEQ ID NO 27
Kan12 without dBTP	GGATAAAATGCTTGATGGTCGGAAGAGGCATAAAGTCCCGTCAGCCAGTT		SEQ ID NO 28
Kan13 without dBTP	GGATAAAATGCTTGATGGTCGGAAGAGGCATAAA-TTCCGTCAGCCAGTT		SEQ ID NO 29
KanR_AEGIS	TAGTCTGACCATCTCATCTGTBACBTCBTTGGCABCGCT-ACCTTTGCCA		SEQ ID NO 23
KanR_normal	TAGTCTGACCATCTCATCTGTAACATCATTGGCAACGCT-ACCTTTGCCA		SEQ ID NO 24
Kan09 with dBTP	TAGTCTGACCATCTCATCTGTAACATCATTGGCAACGCT-ACCTTTGCCA		SEQ ID NO 25
Kan11 with dBTP	TAGTCTGACCATCTCATCTGTCACATCATTGGCAACGCT-ACCTTTGCCA		SEQ ID NO 26
Kan14 with dBTP	TAGTCTGACCATCTCATCTGTAACATCATTGGCCACGCT-ACCTTTGCCA		SEQ ID NO 27
Kan12 without dBTP	TAGTCTGACCATCTCATCTGTAACATCATTGGCAACGCTTACCTTTGCCA		SEQ ID NO 28
Kan13 without dBTP	TAGTCTGACCATCTCATCTGTAACATCATTGGCAACGCT-ACCTTTGCCA		SEQ ID NO 29
KanR_AEGIS	TGTTT-CAGAAACAACTCS-GGCGCBTCGGG-CTTCCCA-TACAAGCGAT		SEQ ID NO 23
KanR_normal	TGTTT-CAGAAACAACTCT-GGCGCATCGGG-CTTCCCA-TACAAGCGAT		SEQ ID NO 24
Kan09 with dBTP	TGTTT-CAGAAACAACTCG-GGCGCATCGGG-CTTCCCA-TACAAGCGAT		SEQ ID NO 25
Kan11 with dBTP	TGTTT-CAGAAACAACTCT-GGCGCATCGGG-CTTCCCA-TACAAGCGAT		SEQ ID NO 26
Kan14 with dBTP	TGTTT-CAGAAACAACTCT-GGCGCATCGGG-CTTCCCA-TACAAGCGAT		SEQ ID NO 27
Kan12 without dBTP	TGTTTTCAGAAACAACTCT-GGCGCATCGGG-CTTCCCAATACAAGCGAT		SEQ ID NO 28
Kan13 without dBTP	TGTTT-CAGAAACAACTCTTGGCGCATCGGGGCTTCCCCATACAAGCGAT		SEQ ID NO 29
KanR_AEGIS	A-GATT-GTC-GCACCS-GASTGCCC-GACBTT-ATCGCG--AGCCCATT		SEQ ID NO 23
KanR_nomal	A-GATT-GTC-GCACCT-GATTGCCC-GACATT-ATCGCG--AGCCCATT		SEQ ID NO 24
Kan09 with dBTP	A-GATT-GTC-GCACCT-GATTGCCC-GACATT-ATCGCA--AGCCCATT		SEQ ID NO 25
Kan11 with dBTP	A-GATT-GTC-GCACCT-GATTGCCC-GACATT-ATCGCG--AGCCCATT		SEQ ID NO 26
Kan14 with dBTP	A-GATT-GTC-GCACCT-GATTGCCC-GACATT-ATCGCG--AGCCCATT		SEQ ID NO 27
Kan12 without dBTP	ATGATT-GTCCGCACCC-GAGTGCCCCGACATTTATCGCGGAGGCCCATT		SEQ ID NO 28
Kan13 without dBTP	TAGATTTGTC-GCACCCTGATTGCCCCGACCTTTATCGCG--AGCCCATT		
KanR_AEGIS	T-ATACCCAT-ATAAB-TCBGCBTCC-ATGTT--GGAATTTAAT-CG--C		SEQ ID NO 23
KanR_normal	T-ATACCCAT-ATAAA-TCAGCATCC-ATGTT--GGAATTTAAT-CG--C		SEQ ID NO 24
Kan09 with dBTP	T-ATACCCAT-ATAAA-TCAGCATCC-ATGTT--GGAATTTAAT-CG--C		SEQ ID NO 25
Kan11 with dBTP	T-ATACCCAT-ATAAA-TCAGCCTCC-ATGTT--GGAATTTAAT-CG--C		SEQ ID NO 26
Kan14 with dBTP	T-ATACCCAT-ATAAA-TCAGCATCC-ATGTT--GGAATTTAAT-CG--C		SEQ ID NO 27
Kan12 without dBTP	TTATACCCATTATAAAATCACCATCCCATGTTTGGGAATTTAAT-CGGCG		SEQ ID NO 28
Kan13 without dBTP	TTATACCCAT-ATAAA-TCAGCATCC-ATGTT-GGAAATTTAATTCG--C		SEQ ID NO 29
KanR_AEGIS	GGCCTC-GACGTTTCCC--GTTGAATATGGCTCATGGTG--	863	SEQ ID NO 23
KanR_normal	GGCCTC-GACGTTTCCC--GTTGAATATGGCTCAT------	810	SEQ ID NO 24
Kan09 with dBTP	GGCCTC-GACGTTTCCC--GTTGAATATGGCTCATGGTG--	849	SEQ ID NO 25
Kan11 with dBTP	GGCCTC-GACGTTTCCC--GTTGAATATGGCTCATGGTG--	849	SEQ ID NO 26
Kan14 with dBTP	GGCCTC-GACGTTTCCC--GTTGAATATGGCTCATGGTG--	843	SEQ ID NO 27
Kan12 without dBTP	GGCCTCCAACGTTTCCCCGGTTGAATATGGCTTCTAGGGTG	872	SEQ ID NO 28
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sgastt 66

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<400> SEQUENCE: 5

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sc 62

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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic

<400> SEQUENCE: 6

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<210> SEQ ID NO 7
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
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<400> SEQUENCE: 7

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<210> SEQ ID NO 8
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic

<400> SEQUENCE: 8

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<210> SEQ ID NO 9
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic

<400> SEQUENCE: 9

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<210> SEQ ID NO 10
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic

<400> SEQUENCE: 10

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<223> OTHER INFORMATION: Synthetic

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<211> LENGTH: 57
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
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<223> OTHER INFORMATION: Synthetic

<400> SEQUENCE: 12

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<210> SEQ ID NO 13
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic

<400> SEQUENCE: 13

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<210> SEQ ID NO 14
<211> LENGTH: 59

<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic

<400> SEQUENCE: 14

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<210> SEQ ID NO 15
<211> LENGTH: 67
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic

<400> SEQUENCE: 15

asttctcbct sgasaacctt atttttgacg aggggaaatt aataggttgt attgasgttg 60

gacgbgt 67

<210> SEQ ID NO 16
<211> LENGTH: 44
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic

<400> SEQUENCE: 16

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<210> SEQ ID NO 17
<211> LENGTH: 62
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic

<400> SEQUENCE: 17

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bc 62

<210> SEQ ID NO 18
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
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<400> SEQUENCE: 18

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<210> SEQ ID NO 19
<211> LENGTH: 68
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic

<400> SEQUENCE: 19

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bcgbcsag 68

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<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic

<400> SEQUENCE: 20

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<210> SEQ ID NO 21
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic

<400> SEQUENCE: 21

caccatgagc catattcaac gg 22

<210> SEQ ID NO 22
<211> LENGTH: 18
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic

<400> SEQUENCE: 22

gtccgtcctg tcagctgc 18

<210> SEQ ID NO 23
<211> LENGTH: 863
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic

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catcgagcat caaatgaaac tgcaasttbt tcatbtcbgg attatcaata ccatattttt 120

gaaaaagccg sttctgtaas gabggagaaa actcaccgag gcagttccat aggatggcba 180

gbtcctggta scggtctgcg attccgacsc gsccbacbtc aatacaacct attaatttcc 240

cctcgtcaaa aataaggttb tcbagsgaga abtcaccatg agtgacgact gaatccggtg 300

agaasggcaa bagsttbtgc atttctttcc agacstgstc bacbggccag ccattacgct 360

cgtcatcaaa atcactcgcb tcbaccaabc cgttattcat tcgtgattgc gcctgbgcga 420

gbcgaaatac gcgatcgctg ttaaaaggac aattacbaac bggaatcgab tgcaaccggc 480

gcaggaacac tgccagcgca tcbacaatbt tstcbcctga atcaggatat tcttctaata 540

cctggaasgc sgtsttsccg gggatcgcag tggtgagtaa ccatgcatcb tcbggbgtbc 600

ggataaaatg cttgatggtc ggbagbggca taaastccgt cagccagttt agtctgacca 660

tctcatctgt bacbtcbttg gcabcgctac ctttgccatg tttcagaaac aactcsggcg 720

cbtcgggctt cccatacaag cgatagattg tcgcaccsga stgcccgacb ttatcgcgag 780

cccatttata cccatataab tcbgcbtcca tgttggaatt taatcgcggc ctcgacgttt 840

cccgttgaat atggctcatg gtg 863

<210> SEQ ID NO 24
<211> LENGTH: 810
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic

<400> SEQUENCE: 24

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accatatttt tgaaaaagcc gtttctgtaa tgaaggagaa aactcaccga ggcagttcca    120
taggatggca agatcctggt atcggtctgc gattccgact cgtccaacat caatacaacc    180
tattaatttc ccctcgtcaa aaataaggtt atcaagtgag aaatcaccat gagtgacgac    240
tgaatccggt gagaatggca aaagtttatg catttctttc cagacttgtt caacaggcca    300
gccattacgc tcgtcatcaa aatcactcgc atcaaccaaa ccgttattca ttcgtgattg    360
cgcctgagcg agacgaaata cgcgatcgct gttaaaagga caattacaaa caggaatcga    420
atgcaaccgg cgcaggaaca ctgccagcgc atcaacaata ttttcacctg aatcaggata    480
ttcttctaat acctggaatg ctgtttttcc ggggatcgca gtggtgagta accatgcatc    540
atcaggagta cggataaaat gcttgatggt cggaagaggc ataaattccg tcagccagtt    600
tagtctgacc atctcatctg taacatcatt ggcaacgcta cctttgccat gtttcagaaa    660
caactctggc gcatcgggct tcccatacaa gcgatagatt gtcgcacctg attgcccgac    720
attatcgcga gcccatttat acccatataa atcagcatcc atgttggaat ttaatcgcgg    780
cctcgacgtt tcccgttgaa tatggctcat                                     810
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<223> OTHER INFORMATION: Synthetic

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tgtaatgaag gacaaaactc accgaggcag ttccatagga tggcaagatc ctggtatcgg    180
tctgcgattc cgactcgtcc aacatcaata caacctatta atttcccctc gtcaaaaata    240
aggttatcaa gtgagaaatc accatgagtg acgactgaat cccgtgagaa tggcaaaagt    300
ttatgcattt ctttccagac ttgttcaaca ggccagccat tacgctcgtc atcaaaatca    360
ctcgcatcaa ccaacccgtt attcattcgt gattgcgcct gagcgagacg aaatacgcga    420
tcgctgttaa aaggacaatt acaaacagga atcgaatgca accggcgcag gaacactgcc    480
agcgcatcaa caatattttc acctgaatca ggatattctt ctaatacctg gaatgctgtt    540
tttccgggga tcgcagtggt gagtaaccat gcatcatcag gagtacggat aaaatgcttg    600
atggtcggaa gaggcataaa ttccgtcagc cagtttagtc tgaccatctc atctgtaaca    660
tcattggcaa cgctaccttt gccatgtttc agaaacaact cgggcgcatc gggcttccca    720
tacaagcgat agattgtcgc acctgattgc ccgacattat cgcaagccca tttataccca    780
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<210> SEQ ID NO 26
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
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<223> OTHER INFORMATION: Synthetic

<400> SEQUENCE: 26

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tgaaactgca atttgttcat atccggatta tcaataccat atttttgaaa aagccgtttc 120

tgtaatgaag gacaaaactc accgaggcag ttccatagga tggcaagatc ctggtatcgg 180

tctgcgattc cgactcgtcc aacatcaata caacctatta atttcccctc gtcaaaaata 240

aggttatcaa gtgagaaatc accatgagtg acgactgaat cccgtgagaa tggcaaaagt 300

ttatgcattt ctttccagac ttgttcaaca ggccagccat tacgctcgtc atcaaaatca 360

ctcgcatcaa ccaaaccgtt attcattcgt gattgcgcct gagcgagacg aaatacgcga 420

tcgctgttaa aaggacaatt acaaacagga atcgaatgca accggcgcag gaacactgcc 480

agcgcatcaa caatattttc acctgaatca ggatattctt ctaatacctg gaatgctgtt 540

tttccgggga tcgcagtggt gagtaaccat gcatcatcag gagtacggat aaaatgcttg 600

atggtcggaa gaggcataaa ttccgtcagc cagtttagtc tgaccatctc atctgtaaca 660

tcattggcaa cgctaccttt gccatgtttc agaaacaact cgggcgcatc gggcttccca 720

tacaagcgat agattgtcgc acctgattgc ccgacattat cgcaagccca tttataccca 780

tataaatcag catccatgtt ggaatttaat cgcggcctcg acgtttcccg ttgaatatgg 840

ctcatggtg 849

<210> SEQ ID NO 27
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<213> ORGANISM: Artificial Sequence
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<223> OTHER INFORMATION: Synthetic

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ctgcaattta ttcatatcag gattatcaat accatatttt tgaaaaagct ttttctgcaa 120

tgaccgaaaa aactcaccga ggcagttcca taggatggca agatcctggt atcggtctgc 180

gattccgact cgtccaacat caatacaacc tattatttcc cctcgtcaaa aataaggtta 240

tcaagagaga aatctccatg agtgacgact gaattttgta agaatggcaa aagtttatgc 300

atttctttcc agacttgatc aacaggccag ccattacgct cgtcatcaaa atcactcgca 360

tcaaccaacc cgttattcat tcgtgattgc gcctgagcga gacgaaatac gcgatcgctg 420

ttaaaaggac aattacaaac aggaatcgaa tgcaaccggc gcaggaacac tgccagcgca 480

tcaacaatat tttcacctga atcaggatat tcttctaata cctggaatgc tgtttttccg 540

gggatcgcag tggtgagtaa ccatgcatca tcaggagtac ggataaaatg cttgatggtc 600

ggaagaggca taaattccgt cagccagttt agtctgacca tctcatctgt aacatcattg 660

gccacgctac ctttgccatg tttcagaaac aactctggcg catcgggctt cccatacaag 720

cgatagattg tcgcacctga ttgcccgaca ttatcgcgag cccatttata cccatataaa 780

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<400> SEQUENCE: 28

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tgtaatgaag gagaaaactc accgaggcag ttccatagga tggcaagatc ctggtatcgg 180

tctgcgattc cgactcgtcc aacatcaata caacctatta atttcccctc gtcaaaaata 240

aggttatcaa gtgagaaatc accatgagtg acgactgaat ccggtgagaa tggcaaaagt 300

ttatgcattt ctttccagac ttgttcaaca ggccagccat tacgctcgtc atcaaaatca 360

ctcgcatcaa ccaaaccgtt attcattcgt gattgcgcct gagcgagacg aaatacgcga 420

tcgctgttaa aaggacaatt acaaacagga atcgaatgca accggcgcag gaacactgcc 480

agcgcatcaa caatattttc acctgaatca ggatattctt ctaatacctg gaatgctgtt 540

tttccgggga tcgcagtggt gagtaaccat gcatcatcag gagtacggat aaaatgcttg 600

atggtcggaa gaggcataaa gtcccgtcag ccagtttagt ctgaccatct catctgtaac 660

atcattggca acgcttacct ttgccatgtt ttcagaaaca actctggcgc atcgggcttc 720

ccaatacaag cgatatgatt gtccgcaccc gagtgccccg acatttatcg cggaggccca 780

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cgtttccccg gttgaatatg gcttctaggg tg 872

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<223> OTHER INFORMATION: Synthetic

<400> SEQUENCE: 29

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tgtaatgaag gagaaaactc accgaggcag ttccatagga tggcaagatc ctggtatcgg 180

tctgcgattc cgactcgtcc aacatcaata caacctatta atttcccctc gtcaaaaata 240

aggttatcaa gtgagaaatc accatgagtg acgactgaat ccggtgagaa tggcaaaagt 300

ttatgcattt ctttccagac ttgttcaaca ggccagccat tacgctcgtc atcaaaatca 360

ctcgcatcca ccaaaccgtt attcattcgt gattgcgcct gagcgagacg aaatacgcga 420

tcgctgttaa aaggacaatt acaaacagga atcgaatgca accggcgcag gaacactgcc 480

agcgcatcaa caatattttc acctgaatca ggatattctt ctaatacctg gaatgctgtt 540

tttccgggga tcgcagtggt gagtaaccat gcatcatcag gagtacggat aaaatgcttg 600

atggtcggaa gaggcataaa ttccgtcagc cagtttagtc tgaccatctc atctgtaaca 660

tcattggcaa cgctaccttt gccatgtttc agaaacaact cttggcgcat cggggcttcc 720

ccatacaagc gattagattt gtcgcaccct gattgccccg acctttatcg cgagcccatt 780

ttatacccat ataaatcagc atccatgttg gaaatttaat tcgcggcctc cgacgttttc 840

ccgttgaata tggctcatgg ga 862

What is claimed is:

1. A process for creating a double stranded DNA ligated product having a preselected sequence, said process comprising:

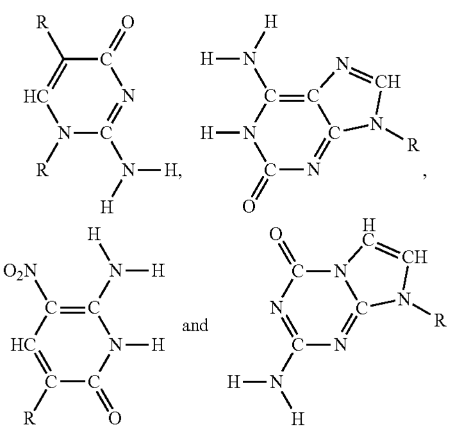
(a) annealing a set of single stranded DNA oligonucleotides having preselected sequences, wherein one or more of said oligonucleotides contain at least two non-standard nucleotides that implement one or more non-standard hydrogen bonding patterns in segments that hybridize to segments of another of said oligonucleotides that contain at least two complementary non-standard nucleotides that implement one or more non-standard hydrogen bonding patterns,

(b) extending the annealed oligonucleotides hybridized at their 3'-ends strand by incubating with 2'-deoxynucleoside triphosphates and a DNA polymerase that is not strand-displacing, and

(c) ligating the extended strands with a ligase.

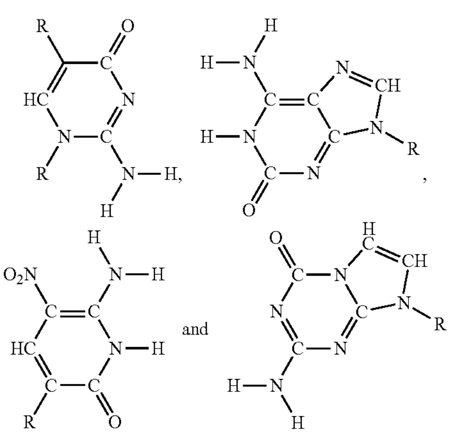
2. The process of claim **1**, wherein said process comprises an additional step, said additional step comprising contacting said ligated product with 2'-deoxynucleoside triphosphates and a DNA polymerase that incorporates standard nucleotides opposite said non-standard nucleotides, copying the strands of said ligated product two or more cycles, and obtaining a product wherein said non-standard nucleotides that implement one or more non-standard hydrogen bonding patterns are replaced by standard nucleotides.

3. The process of claim **1**, wherein said non-standard nucleotides comprise heterocycles selected from the group consisting of



wherein R is the point of attachment between the heterocycles to the said DNA oligonucleotides.

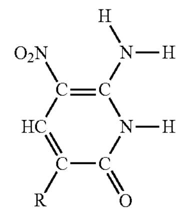
4. The process of claim **2**, wherein said non-standard nucleotides comprise heterocycles selected from the group consisting of



wherein R is the point of attachment between the heterocycles to the said DNA oligonucleotides.

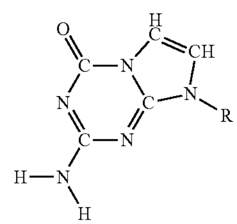
5. The process of claim **1**, wherein said polymerase is Phusion.

6. The process of claim **2**, wherein said process replaces the heterocycle



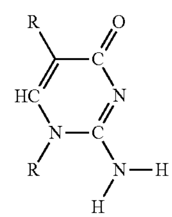
by the heterocycle cytosine, wherein R is the point of attachment between the heterocycles to the said DNA oligonucleotides.

7. The process of claim **2**, wherein said process replaces the heterocycle



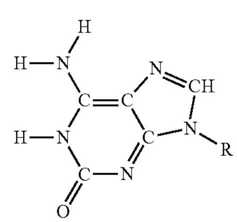
by the heterocycle guanine, wherein R is the point of attachment between the heterocycles to the said DNA oligonucleotides.

8. The process of claim **2**, wherein said process replaces the heterocycle



by the heterocycle thymine, wherein R is the point of attachment between the heterocycles to the said DNA oligonucleotides.

9. The process of claim **2**, wherein said process replaces the heterocycle



by the heterocycle adenine, wherein R is the point of attachment between the heterocycles to the said DNA oligonucleotides.

* * * * *